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<b>(54) Title:</b> CD8 AS AN INHIBITOR OF THE CELLULAR IMMUNE SYSTEM		
<b>(57) Abstract</b> <p>A method for inhibiting CTL activity is described in which soluble CD8 is used as the inhibitor. The method is particularly useful for immunosuppressive therapy in which inhibition of CTL activity is desirable, for example in patients undergoing transplantation.</p>		

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## CD8 AS AN INHIBITOR OF THE CELLULAR IMMUNE SYSTEM

This invention relates to methods for inhibiting immune responses, in particular cellular immune responses involving CD8 positive lymphocytes, using soluble (or non-cellular) forms of CD8. The invention also relates to soluble forms of CD8 and to multimers of the soluble CD8.

### INTRODUCTION

Major histocompatibility complex Class I and II proteins (MHC, or HLA in man) bind peptide antigens and present them on the cell surface for recognition by T lymphocytes expressing a unique T cell receptor (TCR) matching the specific MHC-peptide combination. The transmembrane glycoproteins CD8 and CD4 are characteristic of distinct populations of T lymphocytes whose antigen responses are restricted by class I and class II MHC molecules, respectively. CD8 and CD4 play major roles both in the differentiation and selection of T cells during thymic development and in the activation of mature T lymphocytes in response to antigen presenting cells. CD8 and CD4 are therefore considered to be the main accessory molecules for T cell receptors. Although CD8 and CD4 are immunoglobulin superfamily proteins and determine antigen restriction by binding to MHC molecules, but not to the antigenic peptide, the structural basis for their similar functions appears to be very different. Their sequence similarity is low and whereas CD4 is expressed on the cell surface as a monomer CD8 is expressed as an  $\alpha\alpha$  homodimer or an  $\alpha\beta$  heterodimer.

CD8 expression is characteristic of cytotoxic T lymphocytes (CTL) and important for their progression through the process of 'positive selection' during differentiation in the thymus. In mature cytotoxic lymphocytes the immunological role of CD8 is complex and, as yet, not fully clarified. From the current understanding three putative roles in CTL

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function can be envisaged. Firstly, CD8 plays a role in T cell signalling. Secondly, a significant function of CD8 may be to assist in cell-cell adhesion, tethering the T cell to the antigen presenting cell by increasing the avidity of the interaction. In most, but not all, mature cytotoxic lymphocytes target cell lysis can be blocked by anti-CD8 antibodies. Thirdly, CD8 may serve as a coreceptor for TCR involving a mechanism for collaborative binding of the two receptors to the MHC/peptide ligand. Comparison of the structures of the TCR-MHC and CD8-MHC complexes provide clues for how this cooperativity could work. Although CD8 imposes no detectable conformational changes on the peptide loaded cleft of the MHC molecule, where the TCR binds, it does introduce a shift in the MHC  $\alpha 3$  domain which is similar to a shift introduced by TCR binding. However, analysis involving a human TCR does not indicate that binding is energetically favoured in the presence of CD8.

The T cell coreceptor CD8 is essential for the positive selection of cytotoxic T-lymphocytes (CTL) during differentiation in the thymus and for the ability of most mature CTLs to kill target cells. CD8, expressed on the T cell surface as  $\alpha\alpha$  or  $\alpha\beta$  dimers, contacts the MHC class I molecules or more specifically a nonpolymorphic region of the MHC class I  $\alpha 3$  domain, on antigen presenting cells, thereby increasing the avidity of the T cell for its target. CD8 is also involved in the phosphorylation events leading to T cell activation through the association of the  $\alpha$  chain cytoplasmic tail with p56<sup>lck</sup>.

The analysis of the molecular basis underlying cellular immune responses has suffered from the lack of availability of soluble protein reagents with which to study structural and kinetic aspects of the interactions involved. Recently however a number of groups using expression systems ranging from E.coli to mammalian cells have reported strategies for obtaining soluble TCR. Similarly, a number of groups have reported strategies for obtaining soluble CD8.

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Leahy *et al* (1992) describe the crystallisation of a human soluble CD8 $\alpha$  molecule produced as a secreted disulphide-linked homodimer in CHO cells. The CD8 $\alpha$  contains residues 1 to 146 of the mature protein. In an attempted version employing residues 1 to 114 only,  
5 no detectable secreted protein was produced.

Boursier *et al* (1993) describe the production of a soluble CD8 protein which is expressed as a soluble fusion protein in *E.coli*. One form consisted of residues 1 to 114 of the mature protein, that is the immunoglobulin domain of the protein. Functionality of the soluble protein  
10 is not demonstrated.

Garcia *et al* (1996) describe a soluble CD8 expressed in insect cells. The CD8 molecule contained most of the extracellular part of the CD8 molecule, at least residues 1 to 146 of the mature CD8 protein. Preliminary binding studies are reported.

15 WO 96/22106 and Choski *et al* (1998) disclose peptide fragments of murine CD8 purported to inhibit T cells. The peptides are used at a very high concentration of 100  $\mu$ g/ml. No evidence is given to show whether or not the peptides are actually binding to the MHC molecules.

20

## THE INVENTION

The invention is based on the surprising discovery that soluble forms of CD8 strikingly inhibit CTL activity, both *in vitro* and *in vivo*. Given that CD8 is merely a co-receptor, and given the known low affinity of  
25 CD8 for MHC (Garcia *et al* 1996), this was entirely unexpected. It has been found that binding of soluble CD8 to less than 1% of MHC molecules on target cells is sufficient for inhibition. The effect is explained at least in part by a complete inhibition of the early signalling events in the T cell. This effect bears similarity to the phenomenon of "peptide antagonism" in  
30 which a small ratio of mutated peptide ligand can switch off T cells. It is

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believed that in a similar fashion, the prevention of just a few interactions between the receptors on the T cells and the MHC on the target cells may have a dramatic effect by disrupting or destabilising the multimeric signalling complex in the T cell. In addition, the inhibition effect is  
5 observed to occur strongly at concentrations of soluble CD8 far lower than the peptide concentrations of Choski *et al* (cited above)

A strategy for overexpression in *E.coli* of the extracellular immunoglobulin domain of human CD8 $\alpha$  was devised. Codon usage alterations in the 5' region of the gene were designed so as to prevent the  
10 formation of secondary structures in the mRNA. A fragment of CD8 $\alpha$ , comprising residues 1-120 of the mature protein thus excluding the signal peptide, the cytoplasmic and transmembrane domains and part of the membrane-proximal stalk region, was recovered from bacterial inclusion bodies and refolded to produce a single species of homodimeric, soluble  
15 receptor. Figure 2 illustrates the domain structure of CD8 as described above, and showing other truncations of the CD8 $\alpha$  protein which were expressed but did not fold correctly. HLA-A2 heavy chain and  $\beta$ 2-microglobulin were similarly expressed and refolded with a synthetic peptide antigen corresponding to the *pol* epitope from HIV-1. CD8 $\alpha$ /HLA-  
20 A2 complexes were formed in solution and by co-crystallization with a stoichiometry of one CD8 $\alpha\alpha$  dimer to one HLA-A2-peptide unit.

Data is herein given demonstrating that a fragment of the CD8 $\alpha\alpha$  receptor is capable of inhibiting T cell cytotoxicity, and that mutations of the CD8 $\alpha\alpha$  fragment can modulate that capability. The  
25 inhibitory activity of the soluble CD8 is striking both *in vitro* and *in vivo*. Also described herein is the above strategy for high-level expression of the extracellular immunoglobulin domain of human CD8 $\alpha$  in *E.coli* and for the folding of the CD8 $\alpha\alpha$  dimer as a MHC-binding receptor. Also described is the assembly and crystallisation of the complex between CD8 $\alpha\alpha$  and HLA-

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A2 associated with peptide antigen.

In one aspect, the invention provides a method for inhibiting activity of a T lymphocyte against a target cell, which method comprises contacting the target cell with a soluble form of a CD8 molecule.

5           The CD8 molecule may be any suitable soluble CD8 molecule which has a functional immunoglobulin domain. Generally this will include all or a substantial part of the native CD8 immunoglobulin domain, optionally substituted at one or more amino acid residues, particularly substitutions which increase the binding affinity of the soluble  
10   CD8 for MHC.

          The term "soluble form" is used herein in relation to the CD8 molecule in the manner in which it is conventionally used in the art in relation to cell surface receptors. A soluble form of a cell surface receptor is usually derived from the native form by deletion of the transmembrane  
15   domain. The protein may be truncated by removing both the cytoplasmic and the transmembrane domains, or there may be deletion of just the transmembrane domain with part or all of the cytoplasmic domain being retained. The important thing is that the desired extracellular function of the receptor is retained, which is in this case the MHC-binding capability of  
20   the CD8 immunoglobulin domain. The protein may be modified to achieve the desired form by proteolytic cleavage, or by expressing a genetically engineered truncated or partially deleted form.

          The invention finds particular uses in the treatment of patients requiring immunosuppressive therapy. Such patients include transplant  
25   patients, either awaiting transplant, undergoing transplantation or after transplantation has taken place. Autoimmune diseases and allergies may also usefully be treated by immunosuppressive therapy. One specific example is exacerbated asthma in which T cells come into play as a result of viral infection. Severe damage to the lungs follows and the result is  
30   chronic asthma which can lead to death. The current treatment is with

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corticosteroids which strongly suppress the immune system. A preferable treatment would be one which suppresses the immune system more selectively, such as specific blocking of CTL function by soluble CD8 as described herein.

5                   Thus, the invention provides in another aspect a composition comprising a soluble form of a CD8 molecule together with a pharmaceutically acceptable diluent, excipient or carrier.

                  The invention provides in still another aspect, a particular soluble CD8 protein having the sequence shown in figure 1b, said protein  
10                   folded as a dimer and having the property of inhibiting the action of cytotoxic T cell lymphocytes to kill target cells.

                  In a further aspect the invention provides a soluble CD8 protein which differs from the specific protein defined above, in one or more of the following respects: i) methionine present at the N-terminus; ii)  
15                   one or a few amino acid residues absent from the N-terminus; iii) one or a few amino acid residues added at the N-terminus consisting of part or all of the sequence 'leu - leu - leu - his - ala - ala - arg - pro - ' (the signal sequence); iv) one or a few amino acid residues absent from the C-terminus, but with at least a part of the membrane-proximal stalk region i.e.  
20                   the region defined by amino acids 116 – 120, retained; v) one or a few amino acid residues added at the C-terminus consisting of part or all of the sequence ' - ala - pro - arg - pro - pro -thr - pro - ala'; vi) part or all of the CD8 cytoplasmic membrane peptide sequence added at the C-terminus; vii) conservative variants of one or many amino acid residues which do not  
25                   materially affect the CD8 functionality of the protein; viii) mutations which do alter the CD8 functionality, including those which alter or abolish or reverse the property of inhibiting the action of cytotoxic T cell lymphocytes to kill target cells; ix) the addition of a protein or peptide, at the N or C terminus for the purposes of purification; x) the provision of a label for  
30                   detection; said protein folded as a dimer and having the property of



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affecting the action of cytotoxic T cell lymphocytes to kill target cells. Particularly provided is a protein as described above, in which E replaces N at position 99. Also provided is a protein as described above, in which EE replaces QN at position 54, 55.

5                   More particularly, this aspect of the invention provides a soluble CD8 molecule containing a substantial part of the extracellular region of CD8, including the immunoglobulin domain and a fragment of the membrane proximal stalk region, which CD8 molecule is not disulphide-linked between the two chains of the molecule.

10                   In another aspect, the invention provides a complex of a soluble CD8 protein as herein defined with HLA - A2. A further form of this complex is provided in crystalline form with a stoichiometry of one protein dimer to one HLA - A2 peptide unit.

                  The invention provides, in a still further aspect, a method of  
15                   producing a recombinant protein as herein described, which method comprises the steps of: i) providing a CD8 derived gene suitably modified to allow expression of a protein, essentially corresponding to at least the immunoglobulin-like domain of a CD8 protein, in a bacterium; ii) effecting expression of said CD8 derived gene in said bacterium and recovering the  
20                   expressed protein from a bacterial culture; iii) treating the expressed protein to facilitate its purification and carrying out said purification.

                  Preferably the CD8 derived gene provided at step i) is modified via silent mutations designed to increase expression via the prevention of the formation of a 5' hairpin secondary structure in the expressed mRNA.  
25                   Preferably at step iii) the treatment of the expressed protein involves solubilising the protein and treating the protein so as to cause it to fold into a form resembling its native state, which is then purified. Preferably the CD8 derived gene product corresponds to the immunoglobulin-like and membrane-proximal stalk regions of a CD8 protein.

30                   The soluble CD8 molecules described herein may be in the

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form of a multimer, that is two or more soluble CD8 molecules linked together to produce a bi-functional or multifunctional species. The CD8 molecules may be associated with one another via a linker molecule. Alternatively or additionally the soluble CD8 may be attached to larger  
5 entities such as membrane structures or particles.

Suitable linker molecules include multivalent attachment molecules such as avidin, streptavidin and extravidin each of which has four binding sites for biotin. Thus, biotinylated CD8 molecules can be formed into multimer complexes of CD8 having a plurality of CD8 binding  
10 sites. The number of CD8 molecules in the resulting complex will depend upon the quantity of CD8 in relation to the quantity of linker molecule used to make the complexes, and also on the presence or absence of any other biotinylated molecules. Preferred complexes are trimeric or tetrameric CD8 complexes.

15 Suitable structures for attachment of soluble CD8, optionally already in multimeric form, include membrane structures such as liposomes and solid structures which are preferably particles such as beads. Other structures which may be externally coated with CD8 molecules are also suitable.

20 Multimeric forms of CD8 particularly useful in the method according to the invention include multimers formed by linking biotinylated soluble CD8 via e.g. streptavidin as the linker molecule. One or both chains of the CD8 molecule are biotinylated, conveniently by means of a biotinylation sequence expressed as a tag on the  $\alpha$  or  $\beta$  chain. Also of  
25 particular use are liposome coupled soluble CD8 molecules, in which the CD8 is advantageously itself in the form of a multimer which multimer is then attached to the liposomal membrane by suitable means.

The soluble CD8 will be administered in a manner appropriate for the condition to be treated or prevented. For example, for  
30 prevention of graft rejection one or more injections into the local area

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concerned may be most suitable. On the other hand for an autoimmune disease where the effects are throughout the body it may be more appropriate to inject the soluble CD8 directly into the bloodstream.

Suitable compositions and dosage of soluble CD8 as an immunosuppressive agent can be devised by one of ordinary skill in the art. Two or more doses of a smaller amount of soluble CD8 may be preferable to a single high level dose. Formulations may be for example liquid formulations, or powder formulations such as those designed for delivery by a high velocity needleless delivery device.

The compositions according to the invention may further comprise, or be administered in a treatment regime with, other agents in particular other immunosuppressive agents.

The most commonly used immunosuppressive drugs currently include corticosteroids and more potent inhibitors like, for instance, methotrexate, sulphasalazine, hydroxychloroquine, 6-MP/azathioprine and cyclosporine (Baert and Rutgeerts, 1997; Singer and McCune, 1998). All of these treatments have severe side-effects related to toxicity, however, and the need for drugs that would allow their elimination from, or reduction in use is urgent (McKendry, 1997; Ortiz *et al.*, 1998; Sibilis *et al.*, 1998; Singer and McCune, 1998). Many immunosuppressive drugs have been found to have greater efficacy when used in combination, even when the total dose is lowered (Verhoeven *et al.*, 1998).

Other immunosuppressive drugs include the gentler, but less powerful non-steroid treatments such as Aspirin and Ibuprofen, and a new class of reagents which are based on more specific immune modulator functions. This latter class includes interleukins, cytokines, recombinant adhesion molecules and monoclonal antibodies (for reviews see Baert and Rutgeerts, 1997; Chatenoud, 1998).

sCD8 $\alpha\alpha$  is specific for class I MHC molecules and is therefore expected to inhibit only the response of cytotoxic or memory T

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cells to target cells presenting class I complexes. Many cellular immune responses are of a composite nature, involving class I and class II-restricted T cells. In some situations it may be desirable to use sCD8 $\alpha\alpha$  on its own to suppress unwanted CTL responses. In many situations sCD8 $\alpha\alpha$  may be useful in combination with other immunosuppressive drugs or reagents which suppress other elements of the immune response.

It is envisaged that including sCD8 $\alpha\alpha$  in an immunosuppressive treatment protocol will increase the efficiency of immunosuppression, and particularly, may enable the administered amounts of other drugs, which have toxic or other adverse effects to be decreased.

#### 1. Structure of the CD8 $\alpha\alpha$ receptor.

The human CD8 gene expresses a protein of 235 amino acids. As illustrated in figure 2, the organisation of the protein can be divided into the following domains (starting at the amino terminal and ending at the carboxy terminal of the polypeptide):

- a. signal peptide (amino acids -21 to -1) - this is cleaved off in human cells during the transport of the receptor to the cell surface and thus does not constitute part of the mature, active receptor;
- b. immunoglobulin (Ig) -like domain (approximately amino acids 1-115) - this domain assumes a structure, referred to as the immunoglobulin fold, which is similar to those of many other molecules involved in regulating the immune system, the immunoglobulin family of proteins. The crystal structure of the CD8 $\alpha\alpha$  receptor in complex with the human MHC molecule HLA-A2 has demonstrated how the Ig domain of CD8 $\alpha\alpha$  receptor binds the ligand;
- c. membrane proximal stalk region (amino acids 116-160). This domain is thought to be an extended linker region allowing the CD8 $\alpha\alpha$  receptor to 'reach' from the surface of the T-cell over the top of the MHC

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complex to the  $\alpha 3$  domain of this where it binds. The stalk region is glycosylated and thought to be inflexible. In the context of this invention it should be noted that the stalk region is assumed to play no part in MHC binding;

- 5 d. transmembrane domain (amino acids 161-188). The transmembrane domain anchors the CD8 $\alpha\alpha$  receptor in the cell membrane and is therefore not part of the soluble recombinant protein;
- e. cytoplasmic domain (amino acids 189-214). The cytoplasmic tail of CD8 mediates a signalling function in T-cells through its association
- 10 with p56<sup>lck</sup> which is involved in the T cell activation cascade of phosphorylation events.

The invention encompasses two forms of the CD8 receptor,  $\alpha\alpha$  and  $\alpha\beta$  (EMBL/GENBANK database accession numbers: CD8 $\alpha$ , M27161; CD8 $\beta$ , X13444). The two forms of the receptor are functionally

15 equivalent and no significant differences in the effects of using one or the other for immune inhibition would be expected.

The essential feature of any soluble CD8 protein envisaged is that it contains a correctly folded Ig domain since it is this region that constitutes the binding region for contacting MHC molecules and therefore

20 is responsible for the modulating effect. Variants of the particular CD8 proteins which are the subject of the invention are envisaged and are listed as follows:

- a. Variations of the C-terminal truncation point. It is possible that longer or shorter versions of the receptor could also be stable and
- 25 functional. There is no general rule to predict where the optimal truncation point for a soluble version of a transmembrane protein is. In the case of CD8, the polypeptide could be between 1 and 15 amino acids longer or shorter. However, when shortened at the C terminus the molecule still retains a short fragment of the membrane-proximal stalk region. The
- 30 soluble CD8 could even comprise the cytoplasmic domain, having just the

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transmembrane domain deleted. It is also envisaged that the C-terminus could be fused to peptides or protein domains, such as glutathione-S-transferase for purification purposes, as well known in the art.

b. Variations in the N-terminal truncation point. There is some  
5 uncertainty as to where exactly the signal sequence ends and the Ig domain starts. The N-terminal truncation point could probably be varied somewhat, just like the C-terminal truncation point, without any influence on the functional effect of the protein. It is also envisaged that the N-terminus could be fused to peptides or protein domains, such as  
10 glutathione-S-transferase for purification purposes, as well known in the art.

c. Amino acid substitutions. A large number of conservative amino acid substitutions can presumably be introduced in the protein without causing any significant change in the binding between CD8 receptor and MHC.

15 d. Mutations designed to alter functionality. With the structure of the complex between CD8 $\alpha\alpha$  receptor and a MHC molecule, HLA-A2 (Gao *et al* 1997) it is possible to design mutations predicted to affect the interaction and therefore the immuno-inhibitory effect. The testing of two such mutations is described below, the substitution of residue 99 by a  
20 glutamic acid (designated '99E') and the double substitution of residues 54 and 55 by glutamic acids (designated '54,55E'). These mutations were designed as controls, i.e. the mutations were intended to abolish the binding of the CD8 $\alpha\alpha$  receptor to MHC. Mutant 99E has a residual effect while mutant 54, 55E shows no sign of inhibition (see following T cell lysis  
25 data contained below). Thus the design of mutations with the opposite effect, i.e. which increase the binding between CD8 $\alpha\alpha$  receptor and MHC molecules is also envisaged.

The examples that follow are more clearly described with reference to the figures, of which:

30 Figure 1b shows the amino acid sequence of a soluble

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recombinant human CD8 $\alpha$  protein.

Figure 2 shows CD8 $\alpha$  and truncated proteins expressed in *E.coli*. Schematic presentation of CD8 $\alpha$  illustrating domain organization (top) and the extent of the truncated proteins expressed in bacteria (below). L = leader sequence, Ig = immunoglobulin domain, MP = membrane proximal domain, Tm = transmembrane domain, Cyt. = cytoplasmic domain. Numbers indicate amino acid positions of domain boundaries in relation to the mature protein and the endpoints of proteins expressed in *E.coli*. 'C' signifies the presence and positions of the cysteine residues at positions 143 and 160 involved in forming the interchain disulfide bonds in the CD8 $\alpha\alpha$  dimer. Expression vectors containing the codon information for the illustrated truncated proteins were constructed both with the original codon usage for human CD8 $\alpha$  and with codon usage changes in the 5' end of the insert as described in the Examples.

Figure 19 shows the amino acid sequence of a soluble recombinant mouse CD8 $\alpha$  protein.

The figures are discussed in detail in the Examples.

## EXAMPLES

### Example 1. High level expression of CD8 $\alpha\alpha$ homodimer in *E. coli* and crystallisation.

#### MATERIALS AND METHODS

##### *Cloning of CD8 $\alpha$ cDNA and Construction of *E.coli* Expression Vectors -*

A CD8 $\alpha$  encoding DNA fragment was obtained by the polymerase chain reaction (PCR) using cloned Pfu polymerase (Stratagene) and cytotoxic lymphocyte cDNA as template. The PCR reaction was performed with the sense primer:

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5'-GACTGAGTCGCGGCCGCTGCCACCATGGCCTTACCAGTGACCGCC  
TTG-3' [SEQ ID NO: 1];

and the antisense primer:

5'-TATTCGACTGGATCCTTATACGTATCTCGCCGAAAGGCTGGG-3'

5 [SEQ ID NO: 2].

The PCR product was restriction digested with Eag I and  
BamHI and cloned into pBluescript II KS- (Stratagene) to obtain plasmid  
BJ082. The presence of the full length CD8 $\alpha$  coding sequence was  
verified by doublestranded dideoxy sequencing (USB 70754) using T7  
10 polymerase (Pharmacia). For the construction of bacterial expression  
vectors five PCR products using BJ082 as template were generated which  
encode a methionine followed by the mature immunoglobulin like domain  
of CD8 $\alpha$ . The PCR reactions were conducted with the sense primer  
5'-GGAATTCCATATGAGCCAGTTCGGGTGTCGCCGCTGGATCG-3'  
15 [SEQ ID NO: 3]  
and the antisense primers:

**A:**

5'-CGCGGATCCCTATGCGCCCCCGCTGGCCGGCTCGCCTCTGGGC  
GCAGGGACAG-3' [SEQ ID NO: 4];

20 **B:**

5'-CACCGCGAATTCGGATCCTAAGCGGGTCTACAAGCTTCGGGCTTC  
GCTGGCAGGAAGACC-3' [SEQ ID NO: 5];

**C:**

5'-CACCGCGAATTCGGATCCTAAGCGGGTCTACAAGCTTCTGGCGTC  
25 GTGGTGGGCTTCGC-3' [SEQ ID NO: 6];

**D:**

5'-CACCGCGAATTCGGATCCTAAGCGGGTCTACAAGCTTCGGGCTTC  
GCTGGCAGGAAGACC-3' [SEQ ID NO: 7];

or **E:**

30 5'-GTGGCAAGCTTGGATCCTATGGCGTCGTGGTGGGCTTCGCTG-3'



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[SEQ ID NO: 8].

These PCR products were restriction digested with *Nde* I and *Bam* HI and cloned in vector pGMT7 to obtain expression cassettes for residues A: 1-150 with the cysteine residue at position 143 mutated to serine, B: 1-146, C: 1-120/141-146, D: 1-116/141-146, and E: 1-120. After  
5 sequence verification the vectors were tested for expression (see below) but induction of CD8 $\alpha$  protein expression from all vectors was only detectable by immunoblot analysis employing the monoclonal antibody OKT-8 (ATCC reference CRL8014). Five new PCR fragments encoding  
10 the corresponding proteins were subsequently generated using a modified sense primer,  
5'-GGAATTCCATATGAGTCAATTTCTGTATCACCGCTGGATCG-3'  
[SEQ ID NO: 9], which introduces six silent mutations in the coding region. These PCR fragments were restriction digested with *Nde* I and *Hind* III and  
15 cloned into the vector pGMT7 to obtain plasmids UG195 (A), UG231 (B), UG222 (C), pAÆ3 (D), and BJ112 (E).

*Construction of HLA-A2 Expression Vector -*

A DNA fragment encoding the  $\alpha$ 1,  $\alpha$ 2, and  $\alpha$ 3 domains of  
20 HLA-A\*0201 (HLA-A2), comprising residues 1-276, was obtained by PCR using B cell cDNA as template. The PCR reaction employed the sense primer: 5'-ACATACCCATGGGCTCTCACTCCATGAGGTATTTTC-3' [SEQ ID NO: 10];  
and the antisense primer:  
25 5'-ACATACAAGCTTACGGCTCCCATCTTAAGGTGAGGGGCTTGGG-3'  
[SEQ ID NO: 11]. The PCR product was restriction digested with *Nco* I and *Hind* III and cloned in pET23d+ (Novagen) to obtain plasmid BJ075a in which the sequence of the HLA-A2 fragment was verified by dideoxy sequencing. The plasmid pHN1-  $\beta$ 2m used for expression of the  $\beta$ 2  
30 microglobulin subunit was kindly provided by David N. Garboczi and Don

C. Wiley, Harvard Medical School.

*Expression of CD8 $\alpha$  in E.coli -*

T7-CD8 $\alpha$  expression vectors were transformed into the *E.coli* strain BL21DE3pLysS (Novagen) and from single colonies cultures were grown in TYP medium containing 100  $\mu$ g/ml Ampicillin. Expression from the T7 promoter was induced at approximately OD600 = 0.4 by the addition of IPTG to a final concentration of 0.5 mM and the time-course of induction initially followed for up to eight hours. No increase in protein yields were observed with any constructs after 4 hours following induction by IPTG.

*Large Scale Preparation and Solubilization of CD8 $\alpha$  from Bacterial Inclusion Bodies -*

Bacterial cells were recovered by centrifugation and resuspended in Lysis Buffer (20 ml per liter original bacterial culture) containing 10 mM EDTA, 2 mM DTT, 10mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% v/v Glycerol, 200 mg/l Lysozyme, 1.0mM PMSF. The solution was freeze-thawed and sonicated (Misonix W38S) for a total of 5 min. After mixing with an equal volume of Wash Buffer (0.5% v/v Triton X-100, 10 mM EDTA, 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM DTT, 0.1% v/v Sodium azide) the solution was left on ice for a further 30 min. Inclusion bodies were pelleted by centrifugation at 15,000 rpm in a Beckman JA-20 rotor for 10 min., then resuspended in Wash Buffer (20 ml per liter original culture) using a teflon homogeniser. The recovery and wash steps were repeated four times in total. Following the last spin the pellet was solubilized in Urea Buffer made up as 8 M Urea, 20 mM Tris-HCl (pH 8.0), 10 mM EDTA, 10% v/v Glycerol, 500 mM NaCl, 10 mM DTT and end-over-end mixed overnight at 4°C. Debris was cleared from the inclusion body suspension by centrifugation as described above for 30 min.

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and the cleared suspension stored at -70°C.

*Refolding of the CD8 $\alpha$  Receptor -*

The CD8 $\alpha$  protein (at 10 mg/ml) in 2 ml Urea Buffer was  
5 added to 15 ml Guanidine Buffer (6 M Guanidine hydrochloride, 50 mM  
Tris-HCl (pH 8.0), 10 mM EDTA, 100 mM NaCl, 10 mM DTT) and allowed  
to equilibrate at room temperature for 30 min., then added to 1 litre  
Refolding Buffer made up as 200 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1 M  
L-arginine, 0.1 mM PMSF, 6.5 mM Cysteamine and 3.7 mM Cystamine.  
10 Two more pulses of 20 mg protein were added to the refolding mixture at  
24 hour intervals and the solution stirred at 4°C for 72 hours in total.

*Refolding of HLA-A2/pol -*

HLA-A2 heavy chain,  $\beta$ 2m and peptide (ILKEPVHGV [SEQ  
15 ID NO: 12], synthesized by Oxford Centre for Molecular Sciences and  
corresponding to residues 309-317 of HIV-1 reverse transcriptase) were  
refolded essentially as described with the modification that Guanidine  
Buffer was added to the protein before injecting this into Refolding Buffer  
as described above for folding of CD8 $\alpha$  (David N. Garboczi, personal  
20 communication).

*Purification of Folded CD8 $\alpha$  Receptor -*

The refolding mixture was cleared by centrifugation for  
30 min. at 4,000 rpm in a Beckman J-6B, after which the supernatant was  
25 concentrated to approximately 200 ml in an Amicon 2000 Stirred Cell using  
a Diaflow ultrafiltration membrane with 10 kD pore exclusion size.  
Following this the sample was centrifuged for 30 min. at 15,000 rpm in a  
Beckman JA-20 rotor, then further concentrated to 10 ml in an Amicon  
8400 Stirred Cell. Prior to a two-step purification procedure involving gel  
30 filtration and cation exchange the concentrated sample was filtered through

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a 0.22  $\mu$ m sterile filter. Subsequent preparations of CD8 $\alpha$  have indicated that the centrifugation and filtration steps introduced to clear the protein solution between refolding and concentrating are essential for maintaining correctly folded receptor in solution. The gel filtration step was performed on a Pharmacia Superdex G-75 column equilibrated in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl. Fractions containing CD8 $\alpha\alpha$  homodimer were collected and buffer exchanged into 50 mM MES (pH 6.5) using an Amicon Centriprep-10. The ion exchange step was performed on a Pharmacia Mono-S column from which the protein was eluted with a gradient of 0-1 M NaCl in 50 mM MES (pH 6.5).

#### *Biochemical and Physical Analysis of Purified Proteins -*

Protein concentrations of the folded and purified proteins were determined by Bradford Assay (BioRad). The identity of the folded CD8 $\alpha$  was confirmed by Edman sequencing of the N-terminus. Mass spectroscopic analysis of CD8 $\alpha\alpha$  was performed using a VG Instruments triple-quadrupole atmospheric mass spectrometer fitted with an electrospray ionisation interface. Protein samples of 10  $\mu$ g in 20  $\mu$ l 1:1 v/v acetonitrile/water, acidified by addition of formic acid to a final concentration of 1% v/v, were injected at a flow rate of 20  $\mu$ l/min. The mass spectrometer was calibrated using horse heart myoglobin (Mr 16951.48).

#### *Crystallisation of the CD8 $\alpha\alpha$ /HLA-A2 Complex -*

1  $\mu$ l protein solution containing CD8 $\alpha\alpha$  at 10 mg/ml and HLA-A2/pol at 20 mg/ml were mixed with 1  $\mu$ l reservoir solution from Crystal Screen kits I and II (Hampton Research). Crystallization trials were performed by vapour diffusion from sitting drops on microbridges at room temperature.

## **RESULTS**

*Mutations that Decrease the Tendency for Secondary mRNA Structures in the 5' end of the CD8 $\alpha$  Gene Greatly Increase the Expression Potential in*  
5 *E.coli -*

To construct CD8 $\alpha$  expression vectors for use in a range of expression systems a DNA fragment constituting the entire open reading frame was first generated by the polymerase chain reaction (PCR) using cDNA prepared from a cytotoxic T-lymphocyte cell line as template. A  
10 plasmid containing this fragment was then used as template in a second generation of PCR reactions to synthesize five CD8 $\alpha$  cDNA fragments which were inserted in plasmids containing the T7 promoter. These constructs all encode polypeptide chains in which an initiation codon is followed by the mature CD8 $\alpha$  N-terminal region, comprising the entire  
15 immunoglobulin domain but truncated at different points C-terminally of this (Fig. 2). Constructs with equivalent C-terminal truncations but incorporating the CD8 $\alpha$  signal sequence were tested for expression from a baculovirus promoter in insect cells.

Previously, expression of human CD8 $\alpha$  in insect and CHO  
20 cells has indicated that disulfide bonding of the dimer through Cys143 is critical for the production of a secreted receptor. In contrast, Cys160 which is also involved in covalently linking the membrane-bound receptor was found to prevent expression of soluble CD8 $\alpha\alpha$ . In accordance with this we found that in insect cells CD8 $\alpha$  1-146, 1-120/141-146 and 1-  
25 116/141-146 could be expressed as secreted dimers (data not shown). The requirement for the Cys143 residue for secretion of CD8 $\alpha\alpha$  dimer from insect cells indicates that this residue is essential either for the folding of the protein or for its stability during intracellular transport. These possibilities can not at present be distinguished and since cysteine  
30 residues pose a potential problem when refolding protein *in vitro* constructs

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incorporating or omitting Cys143 were tested in bacteria (Fig. 2).

From plasmids containing inserts with the original human CD8 $\alpha$  codon usage no induction of expression could be detected in *E.coli* as assessed by comparison of whole cell extracts after gel electrophoresis and coomassie staining (Fig. 3, lanes 1-2). In contrast, expression was readily detectable when six codons in the CD8 $\alpha$  gene were altered by silent mutations to make the 5' end of the gene more A/T rich (Fig. 3, lanes 3-4). It has previously been reported that such changes, as would be expected with a lower G/C content and therefore weaker base-pairing potential, decrease the tendency for hairpin formation in the 5' end of the CD8 $\alpha$  mRNA. It appears likely, therefore, that secondary structure formation in the original sequence has a severe adverse effect on the potential for expression in *E. coli*. Expression from the mutated constructs are estimated to yield in excess of 100 mg per litre, an increment of at least two orders of magnitude over the expression level obtained with the unmutated sequence.

*Large-scale Preparation, Initial Purification and Solubilization of Bacterially Expressed Proteins -*

Analysis of cellular fractions from lysed bacteria expressing CD8 $\alpha$  showed that virtually all the protein is deposited in inclusion bodies (Fig. 3) as has previously been observed with bacterially expressed MHC heavy chain and  $\beta$ 2m polypeptides. Purification of the CD8 $\alpha$  inclusion bodies by washing yielded protein estimated to be approximately 90% pure (Fig. 3, lane 5). Similar inclusion body purity was obtained with HLA-A2 and  $\beta$ 2m (not shown). CD8 $\alpha$  inclusion bodies were not soluble in the pH 6.5 buffer used for the MHC chains but adjustment of pH to 8.0, high salt and the addition of 10% Glycerol made it readily soluble.

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*Refolding and Quality Analysis of Soluble CD8 $\alpha\alpha$  Receptor -*

The resolubilized CD8 $\alpha$  polypeptide in reducing and denaturing buffer was diluted into conditions permitting formation of native protein conformation. After allowing folding to proceed for 48 hours soluble protein was concentrated for analysis and purification by gel filtration. Four of the polypeptides expressed and illustrated in Fig. 2 produced mainly aggregate peaks of high molecular weight (data not shown). In contrast to this the elution profile of the 1-120 polypeptide shows one major peak of approximately 30 kDa as expected for the CD8 $\alpha\alpha$  dimer (Fig. 4A). In this elution profile no indication of aggregates is evident. The CD8 $\alpha\alpha$  dimer was further purified by cation exchange on a Mono-S column eluting as a single peak at approximately 175 mM NaCl (Fig 4B). Gel analysis of this peak shows only one band at the expected position for CD8 $\alpha$  (Fig. 3, lane 6).

The identity of the purified CD8 $\alpha$  was verified by two methods. Edman sequencing produced a run of ten amino acids corresponding to the mature N-terminus of CD8 $\alpha$  showing that the introduced methionine residue had been cleaved off in *E.coli*. Electrospray mass spectrometry confirmed the molecular weight of CD8 $\alpha$  (13,464.0) as very close to the theoretical value of 13,463.2 Dalton for residues 1-120 excluding the initiating methionine (Fig. 5). A smaller peak of slightly higher molecular weight is likely to correspond to protein that has not had the methionine residue cleaved off (Fig. 5).

*25 Co-refolding and Soluble Complex Formation between CD8 $\alpha\alpha$  Dimer and HLA-A2/pol -*

To test whether the efficiency of refolding of the component molecules could be increased by complex formation a co-refolding experiment was performed. Gel filtration analysis of the concentrated

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protein reveals that the folding of both CD8 $\alpha\alpha$  and HLA-A2 are impaired and that more aggregates are formed under these conditions than during individual refolding (Fig. 6). A peak is detectable, however, at the expected position corresponding to approximately 75 kDa for the CD8 $\alpha\alpha$  /HLA-A2 complex which is not present in either the CD8 $\alpha$  refolding (Fig. 4A) or in the HLA-A2 refolding (not shown). Analysis of the protein in this peak shows that the components expected in the complex are present in amounts corresponding to one CD8 $\alpha\alpha$  dimer to one HLA-A2 (Fig. 7, lane 1 and 5) indicating that stable complex formation can take place in solution.

#### *Crystallization of CD8 $\alpha\alpha$ in Complex with HLA-A2/pol -*

Crystallization trials, performed with a 2:1 mixture of separately refolded HLA-A2 and CD8 $\alpha\alpha$ , yielded visible crystals under two buffer conditions, 12% PEG 20,000, 100 mM MES pH 6.5 and 15% PEG 6000, 50 mM MES pH 6.0, after approximately seven days at room temperature. One crystal was later diffracted at 2.7 Å resolution. To analyze the composition of the crystal a fragment was washed, resolubilised and analysed by gel electrophoresis (Fig. 7, lane 2). The protein bands present in this sample correspond exactly to those from the individual HLA-A2 and CD8 $\alpha$  (Fig 7, lanes 3 and 4, respectively). Furthermore, the ratio of the CD8 $\alpha$  to the heavy chain and  $\beta$ 2m bands are identical to those found in the sample from the co-refolding peak thought to correspond to the receptor -ligand complex (Fig. 7, lane 1).

#### **Legends for Figures 1 and 3 to 7**

Figure 1a shows cDNA sequence (bottom) and protein sequence (top, one-letter code) of human CD8 $\alpha$ . The extent of the signal peptide, Ig-like domain, membrane proximal domain, transmembrane domain and cytoplasmic domain are indicated.



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Figure 1b shows DNA sequence (bottom) and protein sequence (top, one-letter code) of the expressed fragment of human CD8 $\alpha$ . The extent of the Ig-like domain and membrane proximal is indicated. Bases added on to the DNA sequence in order to encode a translation initiation codon or to enhance expression in *E.coli* are indicated in lower case letters.

Figure 3 shows expression and purification of CD8 $\alpha$ . Approximately 10 $\mu$ g protein samples were analyzed by separation on a 15% SDS-PAGE gel. M = protein size marker; lane 1 = whole lysate of uninduced BL21 cells containing the expression plasmid for residues 1-120 of CD8 $\alpha$  with original codon usage; lane 2 = as lane 1 but induced with 0.5 mM IPTG for 4 hours; lane 3 = lysate of uninduced BL21 cells containing CD8 $\alpha$  expression plasmid with altered codon usage at the 5' end; lane 4 = as lane 3 but induced with 0.5 mM IPTG for 4 hours; lane 5 = purified inclusion body fraction from cells as in lane 4; lane 6 = refolded and purified by gel filtration and ion exchange. The molecular weight of the size markers and the expected position of CD8 $\alpha$  are indicated.

Figure 4 shows purification of CD8 $\alpha$ . A, Superdex G-75 gel filtration profile of concentrated CD8 $\alpha$  refolding. Numbers over the profile indicate the elution points of molecular weight standards. B, NaCl elution profile from Mono-S column of the main peak from the gel filtration.

Figure 5 shows electrospray mass spectrometry analysis of soluble CD8 $\alpha$  receptor. A, electrospray mass spectrum of CD8 $\alpha$  showing mass-to charge ratio peaks after proton bombardment; B, deconvolution of the spectrum shown in A indicating the molecular weight of the single dominant peak.

Figure 6 shows co-refolding gel filtration profile of CD8 $\alpha$  and HLA-A2. Gel filtration was performed on a Superdex G-200 column. Numbers over the profile indicate the elution points of molecular weight standards. The protein components of the obtained peaks are indicated

over the arrows.

Figure 7 shows SDS-PAGE analysis of co-refolded and co-crystallized CD8 $\alpha$  and HLA-A2. Lanes 1-4 and M were stained with Coomassie Blue, lane 5 was silver stained. Lane 1 and 5: co-refolded CD8 $\alpha$ , HLA-A2,  $\beta$ 2m and peptide from peak indicated in Figure 5; lane 2: resolubilized crystal; lane 3: HLA-A2; lane 4: CD8 $\alpha$ , M: protein size markers as indicated. The bands corresponding to A2 heavy chain (A2 HC),  $\beta$ 2m and CD8 $\alpha$  are indicated with arrows.

## 10 DISCUSSION

### *Expression of CD8 $\alpha$*

For expression of CD8 $\alpha$  we found the yields obtained from insect cells insufficient for large-scale purification and therefore focussed on expression in *E.coli*. In bacteria three parameters generally appear to be the main determinants of the expression level which can be achieved for a foreign protein. These are the tendency for secondary structure in the mRNA, the degree to which the codon usage conforms to bacterial preference, and the toxicity for the host of the expressed protein. In the case of CD8 $\alpha$ , six silent codon changes, designed to decrease base-pairing potential close to the N-terminus, increased the expression level by more than two orders of magnitude to a level constituting approximately 30% of the total protein. The high expression levels and the ease with which the protein can be purified from inclusion bodies made bacterial expression the preferred choice for further studies.

25

### *Refolding of CD8 $\alpha$*

The refolding efficiency of CD8 $\alpha$  was found to depend on the buffer conditions used to solubilise the protein from inclusion bodies. In particular the maintenance of a high redox potential, high ion strength and the addition of strongly denaturing agents before refolding dramatically

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- 25 -

improves the yield of the CD8 $\alpha\alpha$  dimer. Analysis of the folded receptor preparation by gel filtration shows virtually no presence of aggregates and after cation exchange the preparation appears to be completely homogeneous and stable for several months at 4°C.

5

*Analysis of the CD8 $\alpha\alpha$ -HLA-A2 Complex Formed in Solution and by Crystallization*

The estimated molar ratios of the polypeptide bands representing HLA-A2 assembly and CD8 $\alpha\alpha$  are identical in the complex  
10 formed in solution and in the crystal but the stoichiometry indicated by the band intensities is somewhat unexpected. Because CD8 has been detected on the T cell surface as a dimer it has been speculated that CD8 would bind two MHC molecules and that a bivalent stoichiometry could be involved in T cell-target cell ligation and T cell activation. However, the gel  
15 analysis of the complexes, taking into consideration that CD8 $\alpha$  stains weaker with coomassie than does  $\beta$ 2m, are more indicative of a 1:1 HLA-A2:CD8 $\alpha\alpha$  ratio and this has been confirmed by the crystal structure. Based on the crystal structure steric considerations rule out that more than one CD8 $\alpha\alpha$  dimer can bind the MHC.

20

Recent studies involving cells or soluble murine CD8 and TCR have indicated that a mechanism for cooperative binding to class I MHC may exist for the two receptors. Comparison of the crystal structures of human CD8 $\alpha\alpha$ /HLA and TCR/HLA indicate that both receptors introduce a shift in the  $\alpha$ 3 domain of the HLA heavy chain which could be associated  
25 with an energetic penalty upon binding. Further studies on complexes of MHC Class I with human CD8 and TCR should clarify whether the structural shift introduced in the HLA heavy chain constitutes a mechanism for cooperative binding of these receptors.

**Example 2. Immune-inhibitory effect of the CD8 $\alpha\alpha$  receptor.**

In a standard cytotoxic T cell assay suitable target cells, i.e. cells which express the relevant restrictive MHC, are first incubated with peptide (1 hour) and radioactive chromium. After washing the target cells are exposed to T cells. The T cell, through T cell receptor (TCR) and CD8, recognises the complex of the peptide and MHC on the target cell surface and, if the ligand is appropriate and present in sufficient concentration, the T cell will lyse the target cell. Lysis is assessed by measuring the release of radioactive chromium.

Figure 8 shows the results of a series of experiments conducted as follows:

A human T cell line (enriched and selected), recognising the HIV1 'gag' peptide (SLYNTVATL [SEQ ID NO: 13]) restricted by HLA-A2, was used. A constant number of target cells, 5000, '868' B cells expressing HLA-A\*0201, were used and the number of T cells was varied. 'E:T' represents a ratio of numbers of T cells to target cells. Four E:T ratios were tested. The peptide concentration was varied from  $10^{-11}$  to  $10^{-7}$  M (horizontal axis on the plots). (The units of peptide concentration are  $\log_{10}$ M unless otherwise marked). Four sets of experiments probing the CD8 effect were conducted, these are marked by the legend on the chart as:

'PBS' - incubation with cell medium alone (phosphate buffered saline), as a control;

'CD8 Ab' - incubation with an anti-CD8 monoclonal antibody (100  $\mu$ g/ml), OKT8, which should have some inhibitory effect by binding to CD8 on the T cell surface and blocking the interaction between this and HLA-A2;

'CD8' - incubation in the presence of soluble CD8 receptor, at 100  $\mu$ g/ml;

'CD8 mutant' - incubation in the presence of soluble CD8

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receptor with the 54-55E double mutation at 100 µg/ml.

The assay components for these experiments were:

18 µl 10X peptide

18 µl PBS or PBS with 1mg/ml CD8αα or CD8 OKT8 Ab

5 50 µl Targets (5,000 868 B cells)

100 µl containing 5,000 –50,000 CTL.

The graphs of figure 8 show the results collected after 2 hours incubation.

Figure 9 shows data from the same experiments as Figure 8  
10 except that the data were collected after 6 hours incubation.

The data in figures 8 and 9 demonstrate:

- that more target cells are killed at high peptide concentration which is as expected. At low concentration the density of specific target molecules on the target cell surface is too low to activate the  
15 T cells.

- CD8 antibody which blocks CD8 on the T cells has some inhibitory effect compared to PBS (control).

- Soluble CD8, which must be assumed to bind the MHC on the target cells, has a clear inhibitory effect, even better than that of the  
20 CD8 antibody.

- The effect of the soluble CD8 is specifically related to its binding to MHC as the mutant which has two critical amino acids substituted has no inhibitory effect.

Figure 10 shows the results of a series of experiments  
25 conducted as follows. The same human T cell line recognising the HIV1 'gag' peptide restricted by HLA-A2 was used as in the experiments of Figures 8 and 9. A constant number of target cells and T cells, 5000 of each were used. The peptide concentration was varied from  $10^{-11}$  to  $10^{-7}$  M (horizontal axis on the plots).

30 Five sets of experiments probing the CD8 effect were

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conducted:

'PBS' - incubation with cell medium as a control;

'CD8 A' - incubation in the presence of one preparation of soluble CD8 receptor at 100 µg/ml;

5                'CD8 B' - incubation in the presence of a second preparation of soluble CD8 receptor at 100 µg/ml;

'Mutant 22' - incubation in the presence of soluble CD8 receptor with the 54-55E double mutation at 100 µg/ml;

10               'Mutant 15' - incubation in the presence of soluble CD8 receptor with the 99E mutation at 100 µg/ml.

Results were collected after 2 hours incubation.

The data in figure 10 extend the conclusions drawn from data in figures 8 and 9, demonstrating similar effects of two preparations of soluble CD8 compared to two mutant CD8s. The E:T ratio was 1:1 and a 2  
15    hour assay was performed.

Figure 11 shows experiments performed as described above except that the concentration of soluble CD8 was varied from 0 – 100 µg/ml. The three traces correspond to peptide concentrations of  $10^{-9}$ ,  $10^{-8}$  and  $10^{-7}$  M. The E:T ratio was 1:1 and a 2 hour assay was performed.

20               The data in figure 11 demonstrate that the CD8 inhibition of cell lysis is titratable.

Figure 12 shows data from an experiment similar to those outlined above except that a T cell clone specific for HIV1 peptide ' pol' (VIYQYMDDL [SEQ ID NO: 14]) restricted by HLA-A2 was used. The  
25    targets were 868 B cells. E:T ratio was 5/1, peptide concentration varied between  $10^{-7}$  and  $10^{-5}$  M. 'Lysis' shows the absolute readout, 'specific lysis' shows results corrected for background.

The data in figure 12 demonstrate that the inhibitory effect of CD8 is also observed with a T cell clone.

30               Figure 13 is another titration of CD8 inhibition showing both

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target cell lysis and T cell stimulation as assessed by MIP1 $\beta$  production. MIP1 $\beta$  is a T-cell activation marker which is produced by T cells in amount proportional to their activation state. This demonstrates that the inhibitory effect is correlated to an inhibition of T cell activation. For the lysis  
5 experiment, E:T ratio was 8:1; peptide concentration was 10  $\mu$ M; no peptide control contained 100  $\mu$ g/ml CD8 $\alpha\alpha$ ; subtracted baseline is no peptide/no CD8 $\alpha\alpha$  negative control. For the M1P experiment 10<sup>5</sup> cells per well were used; 10  $\mu$ M peptide was used in assay of total volume 186  $\mu$ l.

Figure 14 shows data from experiments involving a rare T cell  
10 response that is independent of CD8. The experiment is run similarly to those previously described except that a different sample of cytotoxic T lymphocytes were incubated in a killing assay with '868' B cells at a E:T ratio of 5:1 and with varying concentrations of OKT8 CD8 antibody. The legend shows the data collected after 30 min:

15 'PBS' incubation with cell medium as a control  
'600/100/10  $\mu$ g/ml' - concentration of OKT8 CD8 antibody  
used in each incubation dataset

The data in figure 14 demonstrate that the CTL response for this particular sample of T cells cannot be inhibited by CD8 antibody except  
20 at extremely high concentrations, at which any protein would inhibit due to toxic effects. Thus the CTL response for this particular cell type is CD8 independent.

This experiment used a HLA-A2 Pol-restricted CTL line specific for the peptide ILKEPVHGV [SEQ ID NO: 12]. This cell line is  
25 CD8- independent as defined by the lack of inhibition of target lysis when preincubated with anti-CD8 antibody.

Figure 15 shows data from a similar experiment with the same cells incubated with soluble CD8 receptor at a range of peptide concentrations under similar conditions i.e. E:T 5:1, '868' B cells used as  
30 targets. The legend corresponds to:

- 30 -

'PBS' incubation with cell medium as a control

'CD8' incubation with soluble CD8 $\alpha\alpha$  receptor at 100  $\mu\text{g/ml}$ .

The data in figure 15 demonstrate that the CTL response for this cell sample is not inhibited by soluble CD8.

5                    Thus the data in figures 14 and 15, taken together, demonstrate that the soluble CD8 mediated inhibition observed with the CTL responses examined in figures 8 to 13 is not due to an unspecific effect.

10    **Example 3. Effect of sCD8 $\alpha\alpha$  on early protein tyrosine phosphorylation events in CD8- dependent and independent CTL**

HLA matched, antigen pulsed, Epstein-Barr virus transformed, B cells were used as antigen presenting cells (APC). APC were incubated with soluble sCD8 $\alpha\alpha$  for 5 minutes prior to addition of CTL  
15    at an E:T ratio of 10:1. After incubating for 10 minutes at 37°C cells were pelleted and lysed in lysis buffer (Purbo *et al* 1998). Lysate was cleared by centrifuging at 13,000 rpm in a benchtop microfuge and was separated by SDS-PAGE. Gels were western blotted onto nitrocellulose membrane and the membrane subsequently probed with an anti-phosphotyrosine  
20    mAb. Detection of the primary antibody was by horseradish peroxidase (HRP) linked secondary antibody and enhanced chemiluminescence (ECL).

Results are shown in Figure 16. Anti-phosphotyrosine immunoblots of cell lysates from (A) 003 HLA-A2/Gag CTL clone (CD8-  
25    dependent) and from (B) SC6 HLA-A2/Pol CTL line (CD8-independent). Antigen pulsed B cells ( $10^5$ ) were presented to CTL ( $10^6$ ) either in the absence of sCD8 $\alpha\alpha$  (lane 1), in the presence of 200 or 100  $\mu\text{g/ml}$  sCD8 $\alpha\alpha$  (lanes 2 and 3, respectively), or in the presence of 200 or 100  $\mu\text{g/ml}$  of the CD8 $\alpha$  Glu<sub>54</sub>/Glu<sub>55</sub> mutant (lanes 4 and 5, respectively). Resting CTL  
30    (lane 7) and CTL presented with unpulsed B cells (lane 6) are also shown.



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To allow assessment of both overall protein tyrosine phosphorylation and, specifically,  $\zeta$ -chain phosphorylation the upper and lower parts of the immunoblot were exposed for different times as indicated. Only  $\zeta$ -chain tyrosine phosphorylation is shown for the CD8-independent CTL (B).

5                    This experiment allowed evaluation of the intracellular effects on T cell activation (Fig. 16). In the presence of sCD8 $\alpha\alpha$ , protein tyrosine phosphorylation in the 003 Gag/HLA-A2 restricted CTL clone was not induced in response to the index peptide, resulting only in an activation state similar to that observed in response to B cells not pulsed with agonist peptide (Fig. 16A, lanes 2, 3 and 6). Thus, only peptide specific protein tyrosine phosphorylation is affected and the inhibitory effect of the sCD8 $\alpha\alpha$  therefore appears to be limited to signalling through the TCR/CD3 complex. The inhibition of CD3  $\zeta$  chain tyrosine phosphorylation by sCD8 $\alpha\alpha$  indicates that T cell activation is obstructed at the earliest stage of

10                    the signalling cascade, consistent with inhibition of p56<sup>lck</sup> activation. No inhibitory effect on phosphorylation was observed in the presence of the Glu<sub>54</sub>/Glu<sub>55</sub> mutant (Fig. 16A, lanes 4 and 5). In contrast to the observations involving CD8-dependent CTLs, no effect of either sCD8 $\alpha\alpha$  protein could be observed on CD3  $\zeta$  chain phosphorylation (Fig 16B), or on

15                    overall activation (not shown), in the SC6 Pol/HLA-A2 restricted, and CD8-independent, CTL line.

20                   

                    These results show that soluble CD8 receptor, through its specificity for MHC molecules, can function as a potent inhibitor of CD8-dependent CTL activation. Most striking is the observation that the

25                    sCD8 $\alpha\alpha$  completely abolishes early signalling events through the TCR/CD3 complex. This indicates that the soluble protein, at the employed concentrations, binds at a steady-state level which blocks the majority of CD8-MHC interaction sites, or that obstruction of a subset of MHC molecules for CD8 contacts, but not for TCR contacts, can negate

30                    signalling.

**Example 4. Expression and refolding of CD8 $\alpha\alpha$  with mutation of Cysteine 33 to Serine**

In general, the factor which influences protein folding *in vitro* most is the number of cysteine residues in the polypeptide. The higher the number of cysteine residues, the more combinations are possible for the formation of disulfide bridges, both in the same polypeptide (intrachain disulfide bridges) and between two or more polypeptide chains (interchain disulfide bridges). Therefore, mutation of cysteine residues which are not paired in the native protein conformation is almost invariably beneficial to the yields which are obtained when refolding *in vitro*. Most frequently such residues are mutated to serine or alanine residues.

In CD8 $\alpha\alpha$ , the cysteine residue at position 33 of the mature protein is unpaired in the native conformation (Gao *et al.*, 1997; Leahy *et al.*, 1992). This residue has therefore, in order to increase the yields of CD8 $\alpha\alpha$  which can be obtained through *in vitro* folding, been mutated to a serine residue using the following primers in a PCR mutagenesis reaction (Stratagene):

5' - CTG TCC AAC CCG ACG TCG GGC AGC TCG TGG CTC TTC CAG CCG - 3' [SEQ ID NO: 15]

and 5' - CGGCTG GAA GAG CCA CGA GCT GCC CGA CGT CGG GTT GGA CAG - 3' [SEQ ID NO: 16]

25

The residue which alters the cysteine codon to a serine codon is underlined in the primer sequences.

**Example 5. Refolding of CD8 $\alpha\alpha$  in simplified buffer conditions.**

To reduce the costs of refolding CD8 $\alpha\alpha$  and to eliminate

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some of the buffers which may be undesirable for *in vivo* use of the protein, refoldings were tested under simplified conditions.

Virtually identical refolding yields were obtained when the protein is prepared as described in Example 1 prior to refolding (i.e.

- 5 CD8 $\alpha$  at 10 mg/ml in 2 ml Urea buffer was added to 15 ml guanidine buffer and allowed to equilibrate) but refolded in the following buffer conditions:

Refolding Buffer 2:

- 10 0-10 mM Tris-HCl (pH8.0)  
0-0.1 mM PMSF  
6.5 mM Cysteamine  
3.7 mM Cystamine.

15 **Example 6. Expression of CD8 $\alpha$  fused to a biotinylation tag and the production of multimeric CD8 $\alpha$  protein complexes**

The expression vector for the soluble CD8 $\alpha$  gene was also modified to express CD8 $\alpha$  as a fusion to the "biotinylation tag" which is a substrate for the bacterial enzyme Bir A (Schatz, 1993). The following

20 DNA oligo (corresponding to the "antisense" strand) was used together with the sense primer described in Example 1 for a PCR reaction to generate the CD8 $\alpha$  fusion gene:

5' - GGG GGA AGC TTA ATG CCA TTC GAT TTT CTG AGC TTC AAA  
25 AAT ATC GTT CAG ACC ACC ACC GGA TCC TGG CGT CGT GGT GGG  
CTT CGC TGG CAG GAA GAC - 3' [SEQ ID NO: 17]

thus adding the following protein sequence (in one letter code) to the expressed CD8 $\alpha$  polypeptide:

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G S G G G L N D I F E A Q K I E W H [SEQ ID NO: 18].

The tagged CD8 $\alpha$  protein (CD8 $\alpha$ -BT) was expressed at similar levels to CD8 $\alpha$  and refolded as a dimer with similar yield under the same conditions which were used for CD8 $\alpha$ . The functional validity of the tagged protein was demonstrated by Surface Plasmon Resonance experiments in which the CD8 $\alpha$ -BT was immobilised by means of the biotin-streptavidin interaction and was shown to bind soluble class I MHC molecules specifically.

10 In more detail, the CD8 $\alpha$  carrying a biotinylation tag sequence was expressed as described in *E.coli*, refolded, purified and biotinylated with the BirA enzyme (Schatz, 1993). The biotinylated sCD8 $\alpha$  was immobilised on the surface of a Biacore streptavidin-coated flowcell producing a change in the refractive index of 3700 response units (RU). The monoclonal antibody OX68 was immobilised on a different flowcell producing a change in the refractive index of 3400 RU. The biotinylated sCD8 $\alpha$  was passed in succession over the two flowcells, the OX68 antibody-coated flowcell serving as control for the CD8-coated flowcell. The soluble form of the MHC complex HLA-A2 folded with the Pol peptide of HIV-1 (HIV-1 polymerase residues 476-484 with the peptide sequence in one-letter code: ILKEPVHGV [SEQ ID NO: 12]) was flowed over the surfaces at the concentrations indicated in Figure 17. The profiles of the changes in RU from the two flowcells were overlaid (Figure 17). At every concentration, the change in RU was higher in the flowcell coated with sCD8 $\alpha$  than in the flowcell coated with OX68. The respective profiles from the two flowcells are indicated at the four highest concentrations of HLA-A2 complex. As shown in Figure 17, specific binding to the tagged, biotinylated and immobilised CD8 was observed. Thus, this form of CD8 can be immobilised as proposed with its functional integrity intact and is therefore suitable for the production of multimeric CD8 complexes.

Multimeric CD8 complexes can be expected to bind with higher stability to antigen presenting cells than the CD8 $\alpha\alpha$  dimer. The biotinylated CD8 $\alpha\alpha$ -BT protein could, for instance, be made as a tetramer of dimers by attachment to avidin or streptavidin, or attached to various  
5 types of avidin- or streptavidin-coated beads, or bound to the surface of liposomes made with derivatised lipids. In this way CD8 higher-order multimerisation particles would be obtained which would bind with significant avidity to antigen presenting cells and therefore act as efficient suppressors of the cytotoxic T cell response.

10

#### **Production of CD8 tetramers and CD8-coated beads.**

In order to tetramerise the biotinylated CD8 $\alpha\alpha$ , extravidin (Sigma) is added at a 1:4 molar ratio. Fluorescently labelled extravidin is used for cell-labelling experiments. A step-wise addition is employed to  
15 achieve saturation of the extravidin, allowing for some incompleteness in the biotinylation reaction and some inaccuracy in the protein determinations. 15 minutes on ice is allowed between each addition of extravidin for binding, followed by at least overnight at 4°C after the final addition. Tetramerisation is confirmed by gel filtration of a small sample of  
20 the solution on a calibrated Superdex 200 column (Pharmacia). CD8 tetramer solution is then stored at 4°C in the presence of protease inhibitor cocktail and 0.05% sodium azide.

A similar approach can be used to coat various types of beads or other solid supports with soluble CD8. Avidin/streptavidin-coated  
25 beads can be obtained from commercial sources (for instance, Dynabeads from DYNAL, Oslo, Norway, or MACS from Miltenyi Biotec Ltd., Bergisch Gladbach, Germany) and are available in a wide range of sizes from approximately 4.5 $\mu$ m-65nm in diameter. Immobilisation of MHC-peptide complexes on Dynabeads through Biotin-Streptavidin has previously been  
30 described (Vessey *et al.*, 1997). Purified biotinylated protein is incubated

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with streptavidin-coated beads for a period of time e.g. 30 mins at 4°C after which the beads are washed to remove unbound protein. These MHC-peptide coated beads elicited an antigen-specific response when used to stimulate a cell line expressing TCR. Similarly, tetramers of CD8, or  
5 monomeric biotinylated soluble CD8, can be immobilised on avidin/streptavidin-coated beads, or non-biotinylated CD8 can be immobilised by means of anti-CD8 antibody coating or by direct chemical crosslinking or by other appropriate means.

#### 10 **Production of liposomes and drug packaging**

Lipids and other components, sterile and endotoxin tested, are commercially available from a number of sources, for instance from Sigma Chemical Company or Avanti Polar Lipids Inc., USA.

Liposomes are prepared from a mixture of vesicle-forming  
15 lipids and biotinylated vesicle-forming lipids. A variety of suitable methods exist for liposome formation. Biotinylated CD8 is then linked to the exterior of the liposomes via a suitable linking agent such as avidin, streptavidin or extravidin. Detectable labels and/or reagents such as therapeutic agents, if desired are incorporated into the membrane itself or entrapped in the  
20 aqueous volume within the membrane.

#### **Example 7. Residues which might be mutated in order to alter the affinity of CD8 $\alpha\alpha$ for MHC.**

The affinity of the CD8 $\alpha\alpha$  dimer for MHC may possibly be  
25 increased by substitution of one or more of the residues which form contacts to MHC. An increased affinity may increase the ability of the sCD8 $\alpha\alpha$  to inhibit cytotoxic T cell responses since the occupancy of the sCD8 $\alpha\alpha$  molecule on the MHC molecules on the surface of antigen presenting cells would be expected to be higher. It is envisaged that  
30 substitutions of one or more from the following list of residues, numbers

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referring to the amino acid positions in the mature CD8 $\alpha$  protein sequence, could increase the affinity of CD8 $\alpha\alpha$  for MHC:

Arginine 4	Serine 31
5 Lysine 21	Serine 34
Valine 24	Tyrosine 51
Leucine 25	Glutamine 54
Leucine 26	Asparagine 55
Serine 27	Aspartate 75
10 Asparagine 28	Leucine 97
Proline 29	Asparagine 99
Threonine 30	Serine 100

See (Gao *et al.*, 1997) for list of contact residues as deduced from the CD8 $\alpha\alpha$ /HLA-A2 structure.

15 Substitution of amino acid residues is routine and can be carried out using known techniques. Substituted CD8 $\alpha$  can then be tested for binding to MHC using techniques described herein, and/or tested for a T cell inhibition effect by incorporating it into a CD8 dimer and performing a cytotoxic T cell assay, for example as described in Example 2.

20

#### **Example 8. Murine soluble CD8 $\alpha\alpha$ for animal experiments.**

The homology between CD8 and the regions of MHC molecules contacted by CD8 makes it possible to test the immuno-inhibitory effect of the human CD8 in animal experiments (see also

25 Example 10. However, it is envisaged that a murine sCD8 $\alpha\alpha$  will be employed for certain experiments in mouse systems. The murine sCD8 $\alpha\alpha$  may be of similar design to the human molecule described herein. The murine molecule will be particularly useful for the study of the long-term

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effects of administering sCD8 $\alpha$ . Although the human and murine CD8 $\alpha$  molecules are very similar in sequence, secondary immune reactions directed against the human molecule could affect its function during long-term studies in the mouse.

5 A murine CD8 $\alpha$  gene for expression in *E.coli*, and refolding into a soluble dimeric form similar to the human sCD8 $\alpha$ , has been generated by the polymerase chain reaction method (PCR) employing murine CTL cDNA as template and the following primer sequences designed to match the murine CD8 $\alpha$  (Lyt-2; (Nakauchi *et al.*, 1985)):

10

sense primer:

Nde I

M K P Q A P E L R I

15 5' - GAG GAG GAG CAT ATG AAA CCA CAA GCA CCT GAA CTA CGA ATC

F P K K M D A

TTT CCA AAG AAA ATG GAC GCC -3' [SEQ ID NO: 19]

20 antisense primer:

HindIII

5' - ggg gag gga agc tta ctt ggt agt agt aga gtt cac -3'

[SEQ ID NO: 20]

25 The bases constituting restriction sites, introduced for cloning purposes, and bases altered in the sense primer in order to increase expression in *E.coli* are underlined.

Figure 18 shows the cDNA sequence, the protein sequence, and the domain organisation of the murine CD8 $\alpha$  (database accession no. M12052). Figure 19 shows the DNA sequence of the DNA fragment

30 produced in the PCR reaction and the deduced protein sequence of the



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recombinant, soluble murine CD8 $\alpha$ . It is envisaged that variations of the soluble murine CD8 $\alpha$  as described herein for the soluble human CD8 $\alpha$  may also be generated and used in animal experiments. Such variants may be for example one or a few amino acids longer or shorter at the N-terminus, or one or a few amino acids longer or shorter at the C-terminus, than the protein depicted here. Other silent mutations (that is, DNA mutations which do not alter the protein sequence) in addition to or instead of those indicated herein may also be introduced in the 40 most 5' bases of the expression cassette shown in Figure 19 with the aim of increasing expression in *E.coli*.

Production of proteins in which the unpaired cysteine residue indicated in Fig. 18 and Fig. 19 is mutated in order to increase refolding efficiency is also envisaged.

#### Legends to Figures 18 and 19

Figure 18. cDNA sequence of mouse CD8 $\alpha$  (bottom sequence) with protein sequence (on top) indicated in one-letter code. The predicted extent of the signal sequence, immunoglobulin-like domain, membrane-proximal "stalk" region, transmembrane domain and cytoplasmic domain are indicated over the protein sequence (Nakauchi *et al.*, 1985; Nakauchi *et al.*, 1987). The two cysteine residues which are believed to form the intrachain disulfide bond are indicated with •. The cysteine residue which is believed to be unpaired, and which may be mutated in order to increase protein yield as suggested for the human CD8 $\alpha$  (see Example 4) is indicated with #.

Figure 19. DNA sequence (bottom sequence) of cassette for expressing mouse CD8 $\alpha$  in *E.coli*. The protein sequence (on top) is indicated in one-letter code. The two cysteine residues which are believed to form the intrachain disulfide bond are indicated with •. The cysteine residue which is believed to be unpaired, and which may be mutated in

order to increase protein yield, as suggested for the human CD8 $\alpha$  (see Example 4), is indicated with #. Recognition sites for restriction enzymes with which the cassette, generated by PCR, can be cloned into a suitable expression vector, for instance pGMT7 (Studier *et al.*, 1990), are also  
5 indicated. Bases which are not part of the original DNA sequence, and which have been added to encode a start codon, allow subcloning, or have been mutated to increase bacterial expression, are shown in small letters.

**Example 9. Determination of binding affinity of soluble CD8 to MHC**

10 A surface plasmon resonance (SPR) technique may be used to study the affinity of the interaction between soluble CD8 and MHC class I –peptide complex. This may be useful for example to determine whether a soluble CD8 molecule substituted at one or more amino acid residues has an increased or decreased binding affinity compared to non-  
15 substituted soluble CD8.

SPR studies are performed using a BIAcore™ 2000 (BIAcore AB, St Albans, UK) in HBS (BIAcore AB). HBS contains 10 mM HEPES (pH 7.4), 150 mM NaCl, 3.4 mM EDTA and 0.005% Surfactant P20. Streptavidin (Sigma) is covalently coupled to Research Grade CM5 sensor  
20 chips (BIAcore) via primary amines using the Amine Coupling kit (BIAcore). For coupling, the streptavidin is dissolved in 10 mM sodium acetate (pH 5.5) and injected at 0.5 mg/ml. Immobilisation levels range from 6000 to 11000 Response Units. Biotinylated HLA-A2 and control proteins are then immobilised by injection at 0.05 to 0.15 mg/ml for 0.5 to 10 min over  
25 streptavidin-coupled surfaces. Biotinylated mouse and rat monoclonal antibodies are used as control proteins.

Soluble CD8 which binds to the HLA-A2 will show a larger response when injected over the surface presenting immobilised HLA-A2 than when injected over the control surface. Binding affinity of a soluble  
30 CD8 for HLA can be measured.

**Figure 20. CD8 $\alpha\alpha$  binds specifically to immobilised HLA-A2.**

Similar concentrations ( $\sim 300 \mu\text{M}$ ) of wild type CD8 $\alpha\alpha$  or the CD8 $\alpha\alpha$  mutant Q54E/N55D were injected (solid bars) for 90 s through flow cells with HLA-A2 (*solid trace*) or a control protein (*dotted trace*) immobilised. HLA-A2 presented an influenza matrix peptide (HLA-A2-flu, see Table 1). This experiment was performed at 25°C using a flow rate of 5  $\mu\text{l} \cdot \text{min}^{-1}$  with  $\sim 2000$  RU of HLA-A2 or a control protein immobilised. The high background responses seen in the control flow-cell reflect the high refractive index of the injected CD8 $\alpha\alpha$  samples, a consequence of very high protein concentrations. CD8 $\alpha\alpha$  showed a larger response when injected over a surface presenting immobilised HLA-A2 than a control, indicating specific binding. The CD8 $\alpha\alpha$  variant did not bind, confirming the specificity of this interaction.

**Figure 21. The affinity of CD8 $\alpha\alpha$  binding to HLA-A2.**

The affinity of the CD8 $\alpha\alpha$ /HLA-A2 interaction was determined by equilibrium binding analysis. CD8 $\alpha\alpha$  was injected at the indicated concentrations for 30-60 s through flow cells with either HLA-A2-flu or an irrelevant control protein immobilised. Binding was calculated as the difference between the responses in the HLA-A2-flu and control flow cells. This experiment was performed at both (A) 25°C and (B) 37°C. The solid lines represent non-linear fits of the Langmuir binding isotherm to the data. These yielded  $K_d$  values of 156  $\mu\text{M}$  and 736  $\mu\text{M}$  for (A) and (B), respectively. The insets show Scatchard plots of the same data; the  $K_d$  values shown were obtained by linear regression. The flow-rate was 5-10  $\mu\text{l}/\text{min}$  with  $\sim 9000$  and  $\sim 1900$  RUs of HLA-A2-flu immobilised in (A) and (B), respectively. Similar affinities were measured for CD8 $\alpha\alpha$  binding to two different peptide/HLA-A2 complexes (Table 1).

### The kinetics of CD8 $\alpha\alpha$ binding HLA-A2

Since preliminary analysis indicated that the kinetics of the CD8 $\alpha\alpha$ /MHC-peptide interactions are extremely rapid, data were collected at 0.1 second intervals, the fastest rate possible on the BIAcore. When CD8 $\alpha\alpha$  was injected over HLA-A2 at 25°C, equilibrium was reached within 0.4 seconds, making direct determination of the association rate constant ( $k_{on}$ ) impossible. Dissociation was also extremely fast, with the response returning to baseline within 0.4 seconds of the end of the injection.

Because the dissociation time was of the same order as the time taken to wash protein from the flow cell, the flow-rate was increased to 1.67  $\mu\text{L}\cdot\text{s}^{-1}$ , the maximal rate possible on the BIAcore. At this flow-rate, CD8 $\alpha\alpha$  dissociated with an apparent  $k_{off}$  of 18  $\text{s}^{-1}$ . The rate at which the background response falls in the control flow cell ( $\sim 24 \text{ s}^{-1}$ ) provides an estimate of the washing time. This agrees well with the theoretical value ( $\sim 28 \text{ s}^{-1}$ ) calculated for this flow rate [flow rate  $\div$  flow cell volume ( $\sim 0.06 \mu\text{L}$ )]. The true  $k_{off}$  for the CD8 $\alpha\alpha$ /MHC class I interaction may well be greater than 18  $\text{s}^{-1}$  because rebinding following dissociation, which is commonly seen in BIAcore experiments, could not be excluded. In addition, the  $k_{off}$  at 37°C is likely to be higher than at 25°C because the affinity is  $\sim 5$  fold lower (Table 1). If the  $k_{off}$  is  $\geq 18 \text{ s}^{-1}$  and the  $K_d$  is 126  $\mu\text{M}$  (at 25°C), the  $k_{on}$  can be calculated to be  $\geq 140000 \text{ M}^{-1}\cdot\text{s}^{-1}$ . The  $k_{on}$  is typical of protein-protein interactions, and indicates that the low affinity of the CD8 $\alpha\alpha$ /MHC class 1 interaction is not a consequence of an unusually slow  $k_{on}$ .

**Table 1. Equilibrium binding affinity of CD8 $\alpha\alpha$  binding to peptide/HLA-A2.**

Ligand*	Temp(°C)	Kd ( $\mu$ M)**
HLA-A2-flu	25	159 $\pm$ 18 (n = 5)
HLA-A2-flu	37	629 $\pm$ 151 (n = 2)
HLA-A2-pol	25	126 $\pm$ 34 (n = 7)
HLA-A2-pol	37	712 (n = 1)

- 5 \*HLA-A2-flu: HLA-A2 plus Influenza matrix protein 58-66 (GILGFVFTL [SEQ ID NO: 21]); HLA-A2-pol: HLA-A2 plus HIV-1 polymerase 476-484 (ILKEPVHGV [SEQ ID NO:12]).

\*\*Mean  $\pm$  SD or, for n = 2, mean  $\pm$  range of n independent determinations.

10 **Example 10. Inhibition of CTL priming and activity by sCD8 $\alpha\alpha$  *in vivo*.**

The ability of sCD8 $\alpha\alpha$  to inhibit a cellular immune response *in vivo* was tested by two experiments in the mouse, assaying the influence of sCD8 $\alpha\alpha$  on the response to an epitope encoded in a vaccinia virus (see figure 22 for schematic design). All results are presented as the average  
15 values from five individual determinations of each lysis level.

**Materials and reagents:**

Mice : In both experiments mice of strain Black 6 (wildtype laboratory mouse strain) were used.

20

Virus : Vaccinia virus strain G2 (vvG2) is a recombinant virus strain. Infection of cells with Vaccinia virus generally results in the MHC restricted presentation of a number of vaccinia peptide epitopes which are poorly characterised. Infection with strain vvG2, however, also results in the

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presentation of a peptide epitope, corresponding to amino acids 33-41 (sequence in one-letter code: KAVYNFATC [SEQ ID NO: 22]), from the LCM virus G2 glycoprotein. In mice the G2 epitope is presented by the MHC molecule D<sup>b</sup>.

5

Target cells : MC57 antigen presenting cells (Leist *et al.*, 1987) express mouse MHC molecules K<sup>b</sup> and D<sup>b</sup>.

#### Experiment 1:

10 Mouse 1 (M1) was injected, intravenously, at Day 0 with  $2 \times 10^6$  pfu (plaque forming units) Vaccinia virus strain G2 plus 200  $\mu$ l phosphate buffered saline (PBS, standard physiological saline buffer).

Mouse 2 (M2) was injected, intravenously, at Day 0 with  $2 \times 10^6$  pfu Vaccinia virus strain G2 plus 200  $\mu$ l sCD8 $\alpha\alpha$  (20mg/ml) in PBS  
15 buffer.

There were no apparent side effects in either animal.

To assay CTL activity a modification of a previously described procedure was used (Leist *et al* 1987):

On Day 6, the spleens were removed from the mice and  
20 lymphocytes washed out. Spleen cells were cultured in 24-well plates with conA supernatant. Synthetic peptide corresponding to the G2 33-41 epitope was added to the wells and lymphocytes allowed to proliferate for four days.

On Day 10, T cells were counted and CTL killing assays were  
25 performed as follows. Chromium<sup>51</sup>-labelled MC57 cells were prepulsed with G2 33-41 peptide for one hour, then mixed with the cultured mouse lymphocytes at the indicated effector to target ratios. Chromium<sup>51</sup> counts from wells of MC57 cells lysed by addition of detergent were set at 100% lysis.

30

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Experiment 2:

Mouse 3 (M3) was treated identically to M1 except that injections were performed intraperitoneally.

Mouse 4 (M4) was treated identically to M2 except that  
5 injections were performed intraperitoneally.

Again, there were no apparent side effects.

Table 2.

	E:T	M1 (PBS)	<u>M2 (CD8)</u>	M3 (PBS)	<u>M4 (CD8)</u>
10	21:1	76%	42%	<u>89%</u>	<u>43%</u>
	7:1	73%	35%	<u>66%</u>	<u>30%</u>

In vitro control experiment:

To verify that the CTL response against G2 33-41 is CD8  
15 dependent, and inhibitable by sCD8 $\alpha\alpha$ , the effect of the soluble CD8 was tested in a lysis assay employing the CTL cultured from Mouse 1. As shown in Table 3, the mouse CTL response against G2 33-41 is clearly inhibitable by both anti-CD8 antibody and sCD8 $\alpha\alpha$ .

20 Table 3. Inhibition of M1 CTL response by anti-CD8 mAb YTS 169.4 (Cobbold *et al.*, 1984) and by sCD8 $\alpha\alpha$ . Effector to target ratio (E:T) was 20:1 in all experiments. 100  $\mu$ g anti-CD8 mAb YT62.1 or sCD8 $\alpha\alpha$  in 25  $\mu$ l were added into 200  $\mu$ l assays.

25	Addition	PBS	YT62.1	sCD8 $\alpha\alpha$
	Lysis level	82%	46%	44%

Experiment 3:

Mice 5 and 6 (M5 and M6; control animals) were treated  
30 identically to M3 except that two injections of PBS buffer were

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administered, one five hours prior to infection with vaccinia virus G2, and a further injection 24 hours after the first one.

Mouse 7 and 8 (M7 and M8) were treated identically to M4 except that two injections of sCD8 $\alpha\alpha$  were administered, one five hours  
5 prior to infection with vaccinia virus G2, and a further injection 24 hours after the first one. As with experiments 1 and 2, no apparent side effects were observed.

On day 6, after the cells were washed out of the spleens, half the wells with lymphocyte cultures were grown in the presence of ConA  
10 supernatant while the other half were grown without. This was done to test whether the effects of sCD8 $\alpha\alpha$  were more clearly detectable without the addition of ConA supernatant. The ConA supernatant contains several cytokines and therefore stimulates both specific and unspecific lysis activity, thus amplifying the lysis which is observed after culturing. This  
15 could contribute to obscuring the inhibitory effect of sCD8 $\alpha\alpha$ .

As seen in Table 4, the sCD8 $\alpha\alpha$  has in both animals had a clear inhibitory effect on the proliferation of cytotoxic T cells specific for the G2 peptide. The inhibitory effect is, as expected, slightly clearer in the cultures grown without ConA supernatant.



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Table 4. Lysis levels of four sets of spleen cultures grown from each of M5-M8. Each lysis level was determined in duplicate.

		Exp.1	Exp.2	Exp.3	Exp.4
M5	-ConA	14%	21%	31%	35%
M6	-ConA	21%	20%	33%	45%
M7	-ConA	-1%	-1%	4%	11%
M8	-ConA	-4%	0%	3%	10%
M1	+ConA	19%	28%	25%	33%
M2	+ConA	19%	33%	56%	38%
M7	+ConA	2%	7%	7%	20%
M8	+ConA	7%	12%	21%	24%

5                   The "negative" lysis observed in two of the spleen cultures from M7 and M8 is due to complete inhibition of CTL and the subtraction of "spontaneous lysis" from all of the results. This phenomenon is common in CTL assays.

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### CLAIMS

1. A method for inhibiting activity of a T lymphocyte against a  
5 target cell, which method comprises contacting the target cell with a  
soluble form of a CD8 molecule.
2. The method according to claim 1, wherein the soluble CD8 is  
human CD8.
3. The method according to claim 1 or claim 2, wherein the  
10 soluble CD8 comprises all or a substantial part of the native CD8  
immunoglobulin domain, optionally substituted at one or more amino acid  
residues.
4. The method according to claim 3, wherein the soluble CD8  
further comprises a fragment of the membrane-proximal stalk region of  
15 CD8.
5. The method according to any one of claims 1 to 4, wherein  
the soluble CD8 is provided as a multimer of two or more CD8 molecules.
6. A composition comprising a soluble form of a CD8 molecule  
together with a pharmaceutically acceptable diluent, excipient or carrier.
- 20 7. The composition according to claim 6, for  
immunosuppressive therapy.
8. A multimer of soluble CD8, comprising two or more CD8  
molecules attached to one another via a linker molecule.
9. A protein having the sequence as shown in figure 1b, said  
25 protein folded as a dimer and having the property of inhibiting the action of  
cytotoxic T cell lymphocytes to kill target cells.
10. A protein which differs from that shown in figure 1b in one or  
more of the following respects:
  - methionine present at the N-terminus;
  - 30 • one or a few amino acid residues absent from the

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N-terminus;

- one or a few amino acid residues added at the N-terminus consisting of part or all of the sequence leu - leu - leu - his - ala - ala - arg - pro -;
  - 5 • one or a few amino acid residues absent from the C-terminus; but with at least a part of the region defined by amino acid residues 116 – 120 retained;
  - one or a few amino acid residues added at the C-terminus consisting of part or all of the sequence - ala - pro - arg - pro - pro -thr -  
10 pro – ala;
  - part or all of the CD8 cytoplasmic membrane peptide sequence added at the C-terminus;
  - conservative variants of one or many amino acid residues which do not materially affect the CD8 functionality of the protein;
  - 15 • mutations which do alter the CD8 functionality, including those which increase or abolish the property of inhibiting the action of cytotoxic T cell lymphocytes to kill target cells;
  - the addition of a protein or peptide, at the N or C terminus for the purposes of purification;
  - 20 • the provision of a label for detection;
  - said protein folded as a dimer and having the property of affecting the action of cytotoxic T cell lymphocytes to kill target cells.
11. A protein according to claim 10, which is a soluble CD8 molecule containing a substantial part of the extracellular region of CD8,  
25 including the immunoglobulin domain and a fragment of the membrane-proximal stalk region, which CD8 molecule is not disulphide-linked between the two chains of the molecule.
12. A protein according to claim 10, in which E replaces N at position 99.

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13. A protein according to claim 10, in which EE replaces QN at position 54, 55.
14. A complex of a protein according to any one of claims 9 to 13 with HLA - A2.
- 5 15. A complex as claimed in claim 14 in crystalline form with a stoichiometry of one protein dimer to one HLA - A2 peptide unit.
16. A method of producing the recombinant protein according to any one of claims 9 to 13, which method comprises the steps of:
- 10 i) providing a CD8 derived gene suitably modified to allow expression of a protein, essentially corresponding to at least the immunoglobulin-like domain of a CD8 protein, in a bacterium;
- ii) effecting expression of said CD8 derived gene in said bacterium and recovering of the expressed protein from a bacterial culture;
- 15 iii) treating the expressed protein to facilitate its purification and carrying out said purification.
17. The method of claim 16 wherein the CD8 derived gene provided at step i) is modified via silent mutations designed to increase expression via the prevention of the formation of a 5' hairpin secondary
- 20 structure in the expressed mRNA.
18. The method of claims 16 or 17 wherein at step iii) the treatment of the expressed protein involves solubilising the protein and treating the protein so as to cause it to fold into a form resembling its native state, which is then purified.
- 25 19. The method of any one of claims 16 to 18, wherein the CD8 derived gene product corresponds to the immunoglobulin-like and membrane-proximal stalk regions of a CD8 protein.

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Figure 1a

```
<----- signal sequence ----->
M A L P V T A L L L P L A L L L H A A R
atggccttaccagtgaccgccttgctcctgccgctggccttgctgctccacgccgcccagg

-> <---- Immunoglobulin like domain ----->
P S Q F R V S P L D R T W N L G E T V E
ccgagccagttccgggtgtcgccgctggatcggacctggaacctgggcgagacagtggag

-----
L K C Q V L L S N P T S G C S W L F Q P
ctgaagtgccaggtgctgctgtccaacccgacgtcgggctgctcgtggctcttccagccg

-----
R G A A A S P T F L L Y L S Q N K P K A
cgcggcgcgcgccgagtcacaccttctcctatacctctccaaaacaagcccaaggcg

-----
A E G L D T Q R F S G K R L G D T F V L
gccgaggggctggacaccagcggttctcgggcaagaggttgggggacaccttctgctcctc

-----
T L S D F R R E N E G Y Y F C S A L S N
accctgagcgcgacttccgcccagagagaacgagggctactatcttctgctcggccctgagcaac

-----> <----->
S I M Y F S H F V P V F L P A K P T T T
tccatcatgtacttcagccacttctgctgccggtcttccctgccagcgaagcccaccacgacg

--- membrane proximal domain -----
P A P R P P T P A P T I A S Q P L S L R
ccagcgccgcgaccaccaacaccggcgccaccatcgcgctcgagcccctgtccctgcg

-----
P E A C R P A A G G A V H T R G L D F A
ccagaggcgtgccggccagcgggcgggggcgagtgcacacgagggggctggacttcgcc

-> <---- Transmembrane domain ----->
C D I Y I W A P L A G T C G V L L L S L
tgtgatctctacatctggggcgcccttggccgggacttggtggggtccttctcctgtcactg

-----> <----- Cytoplasmic domain ----->
V I T L Y C N H R N R R V C K C P R P
gttatcaccctttactgcaaccacaggaaccgaagacgtgtttgcaaagtccccggcct
```

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----->  
V V K S G D K P S L S A R Y V \*  
gtgggtcaaatcgggagacaagcccagcctttcggcgagatacgtctaa

Figure 1b

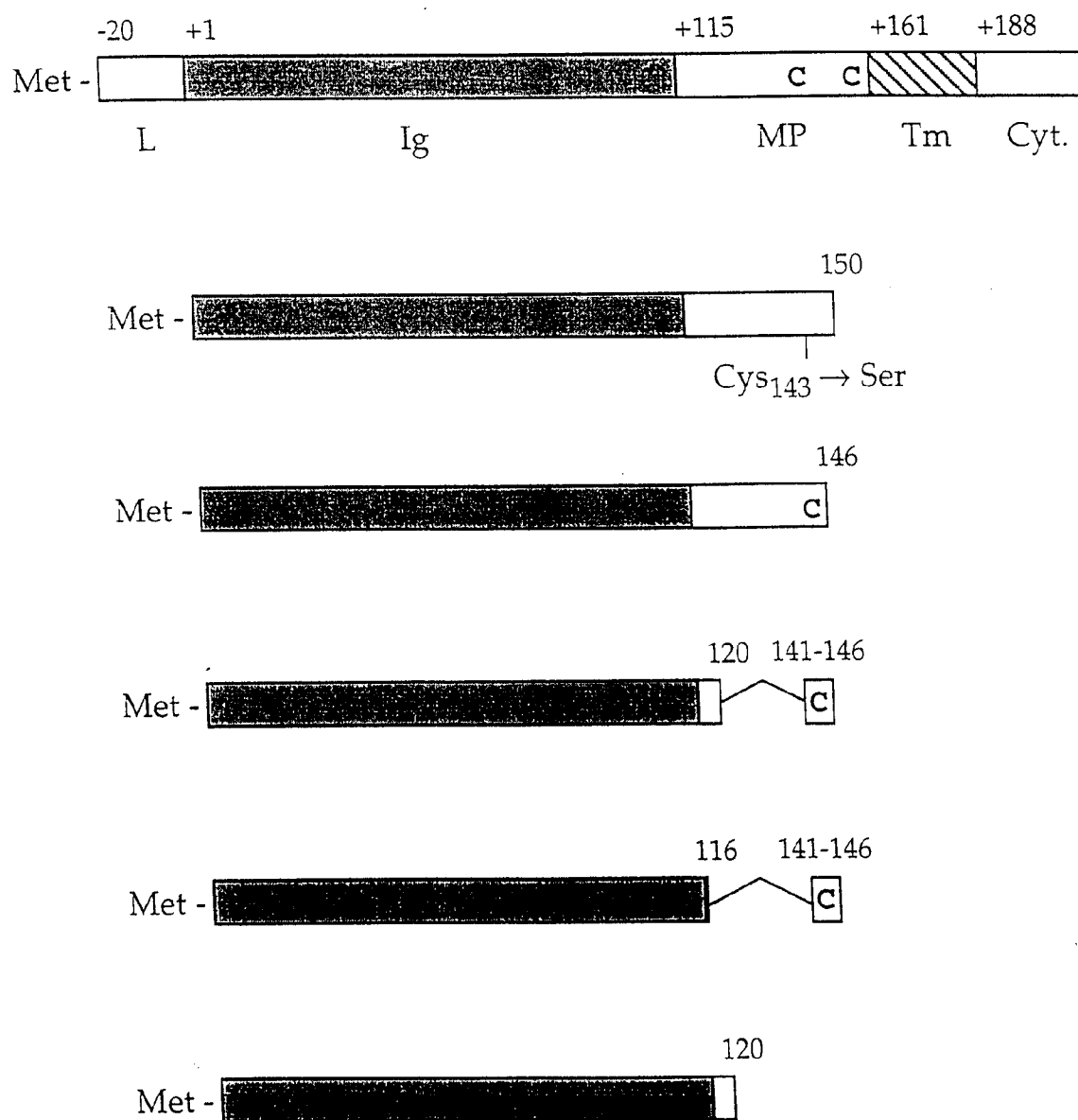
<----- Immunoglobulin like domain ----->  
M S Q F R V S P L D R T W N L G E T V E  
atgAGtCAaTTtCGtGTaTCaCCGCTGGATCGGACCTGGAACCTGGGCGAGACAGTGGAG  
-----  
L K C Q V L L S N P T S G C S W L F Q P  
CTGAAGTGCCAGGTGCTGCTGTCCAACCCGACGTGGGGCTGCTCGTGGCTCTTCCAGCCG  
-----  
R G A A A S P T F L L Y L S Q N K P K A  
CGCGGCGCCGCCAGTCCCACCTTCCTCCTATACCTCTCCCAAACAAGCCCAAGGCG  
-----  
A E G L D T Q R F S G K R L G D T F V L  
GCCGAGGGGCTGGACACCCAGCGGTTCTCGGGCAAGAGGTTGGGGGACACCTTCGTCCTC  
-----  
T L S D F R R E N E G Y Y F C S A L S N  
ACCCTGAGCGACTTCCGCCGAGAGAACGAGGGCTACTATTTCTGCTCGGCCCTGAGCAAC  
-----> <Membrane p.d.  
S I M Y F S H F V P V F L P A K P T T T  
TCCATCATGTACTTCAGCCACTTCGTGCCGGTCTTCCTGCCAGCGAAGCCCACCACGACG  
--  
P \*  
CCATAG

[SEQ ID NO: 23]



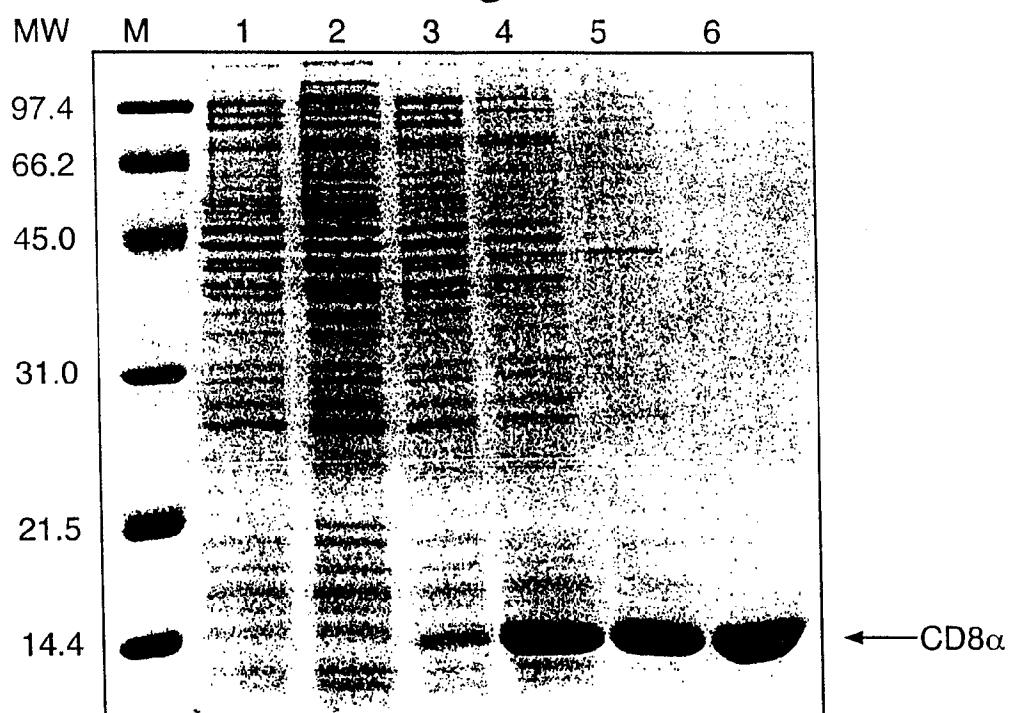
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Fig.2



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Fig.3.



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Fig.4A

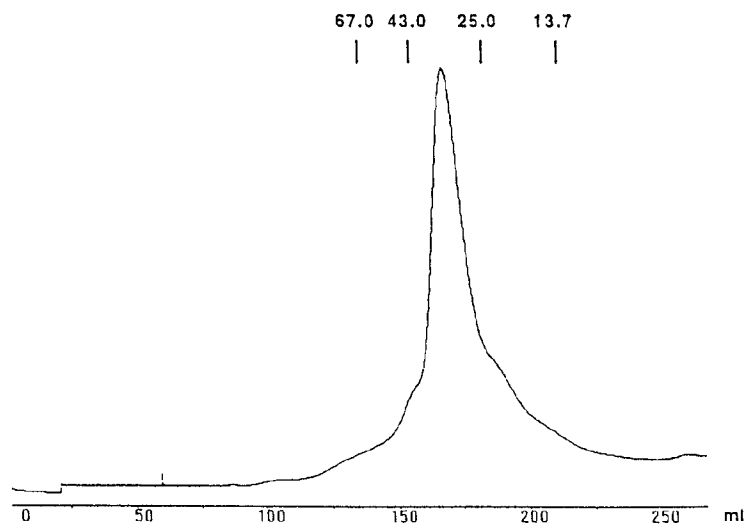
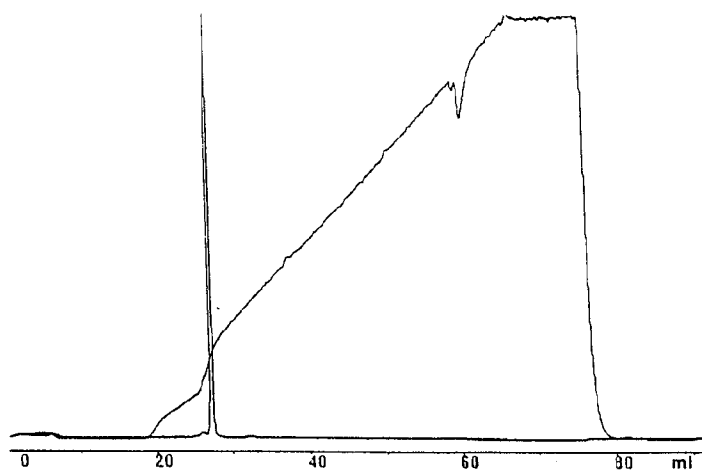


Fig.4B



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Fig.5A

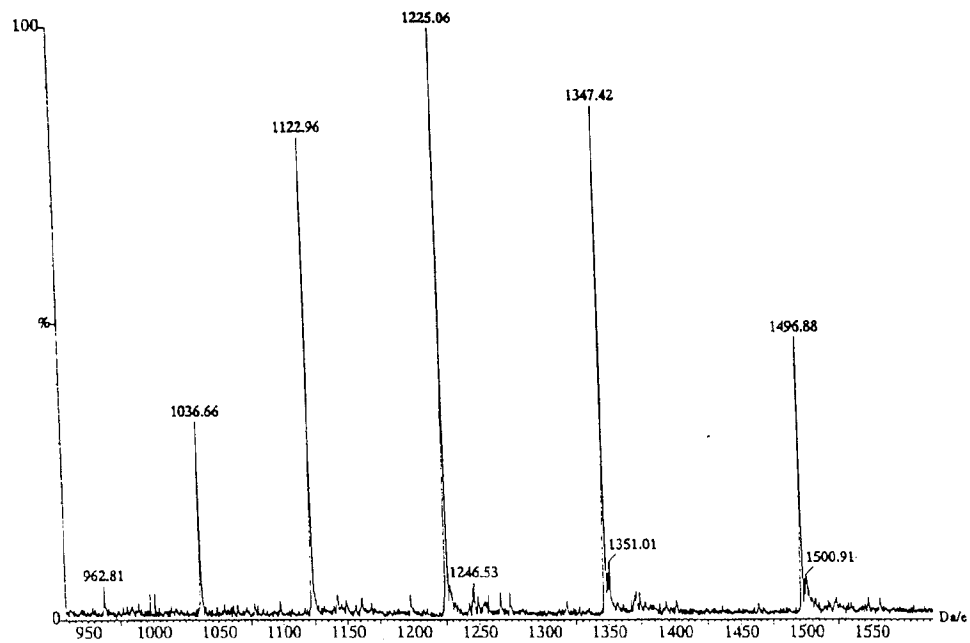
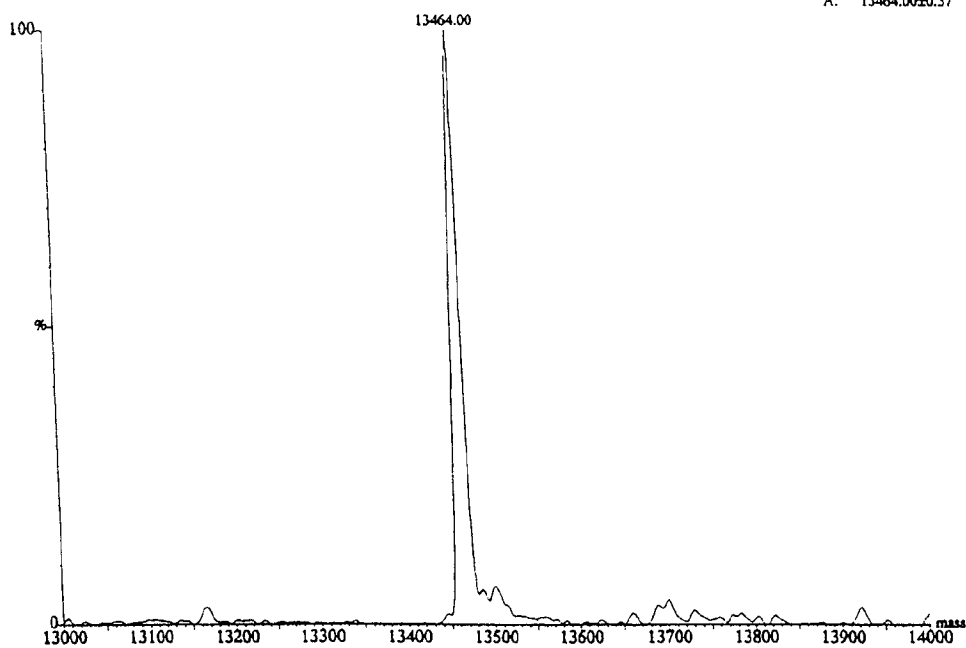


Fig.5B



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Fig.6.

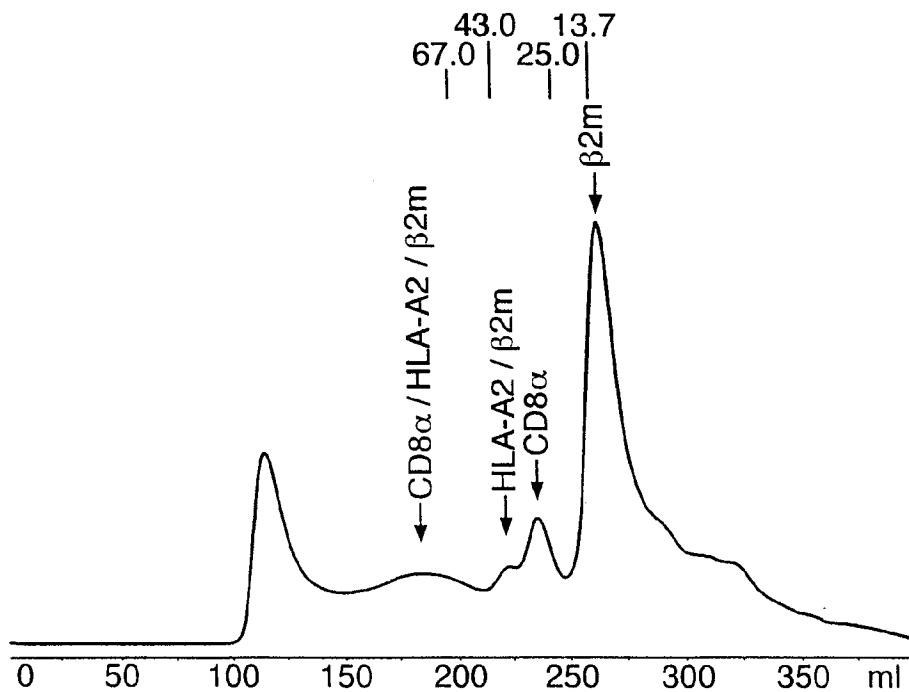
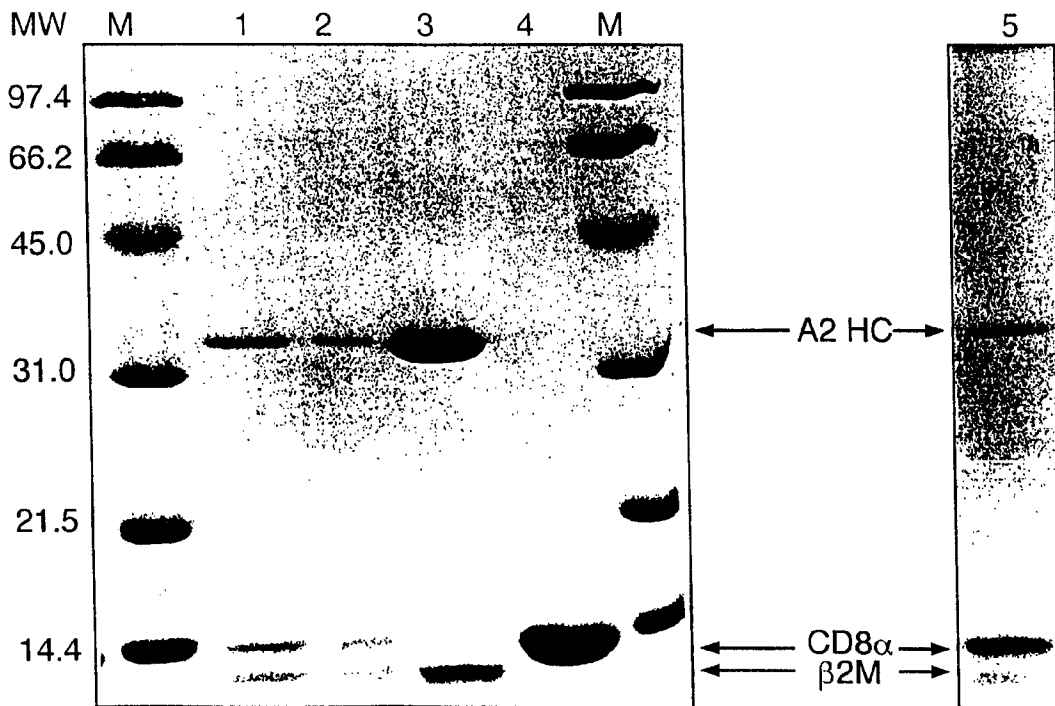
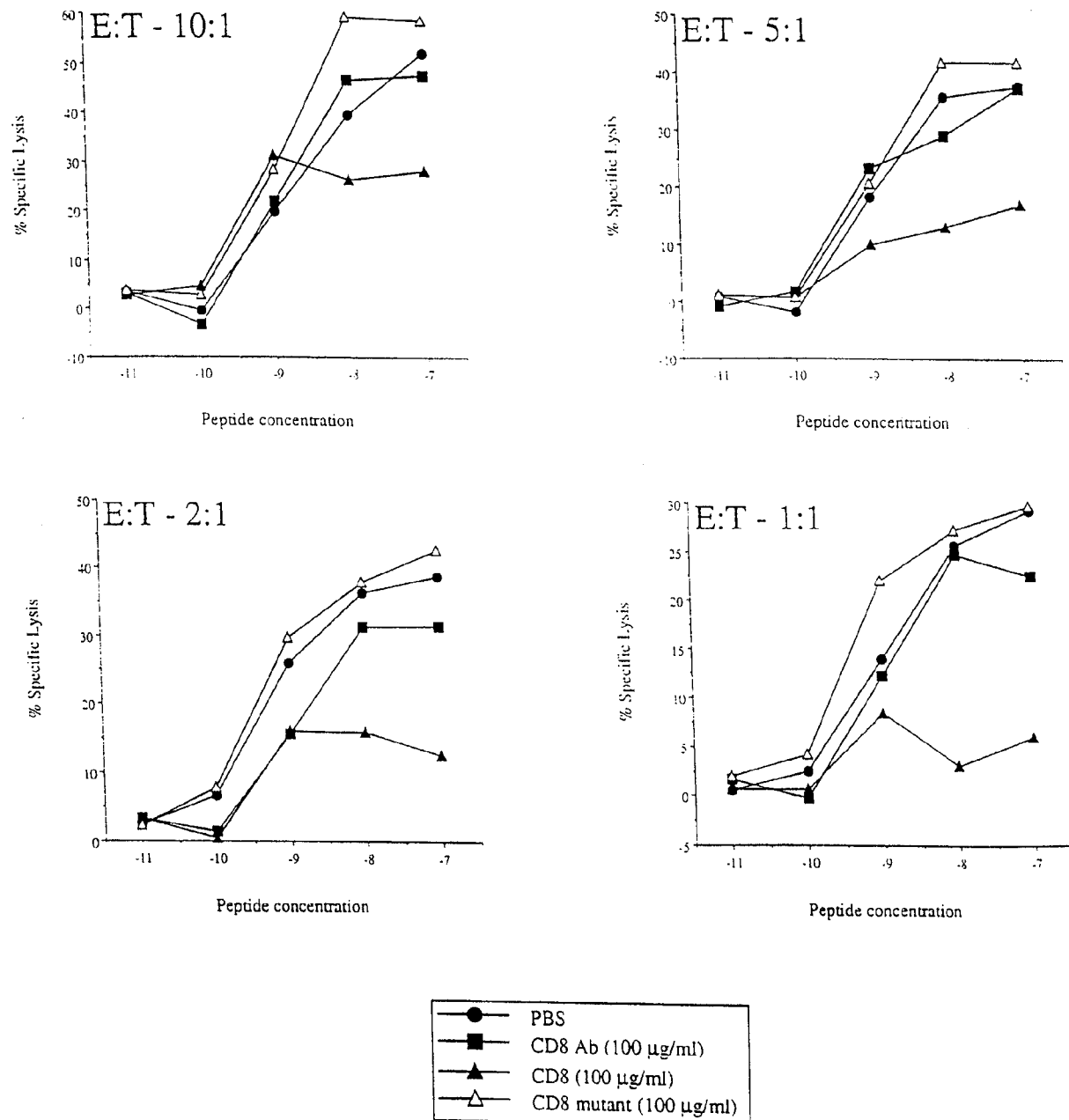


Fig.7.



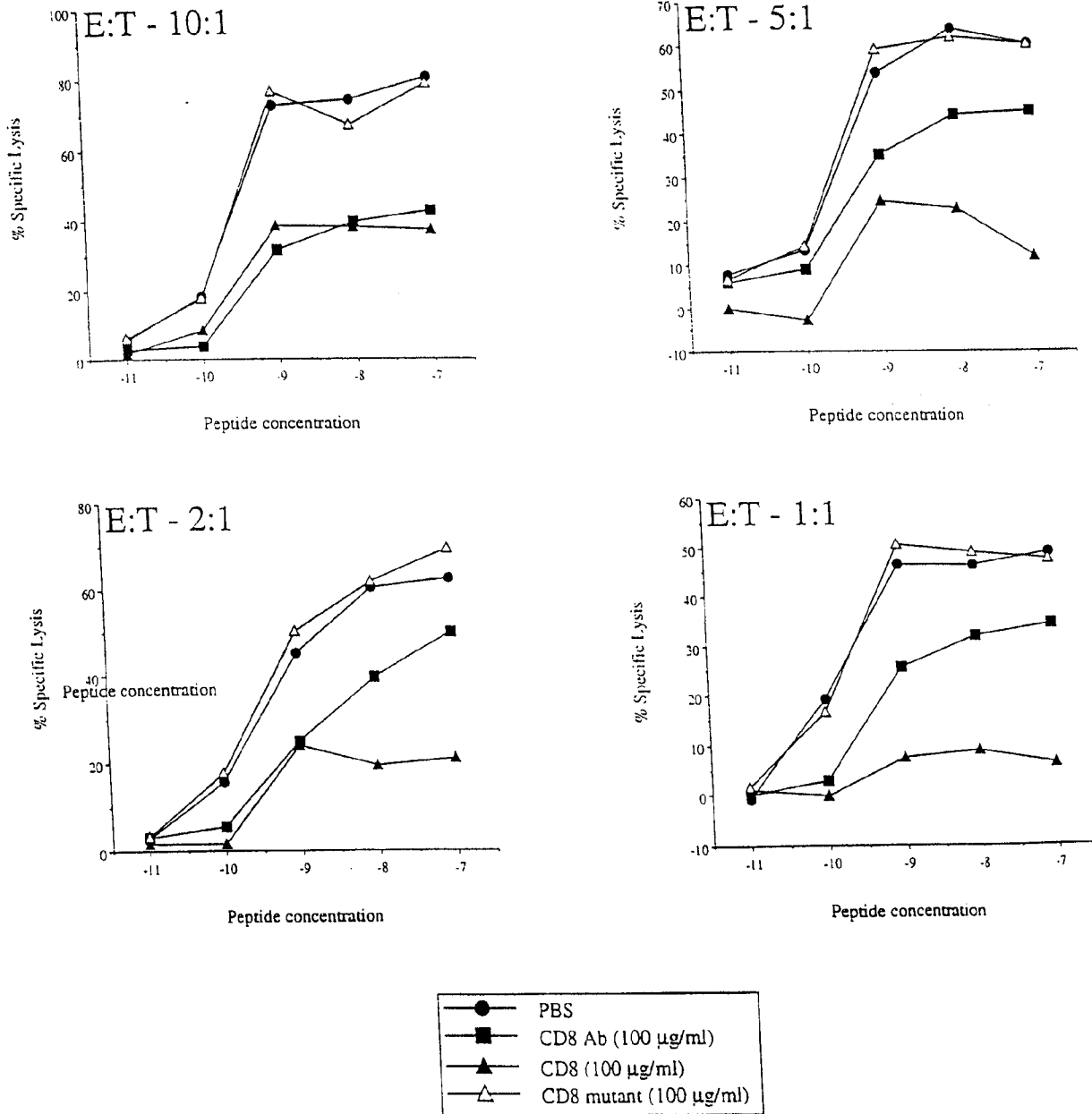
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Fig.8



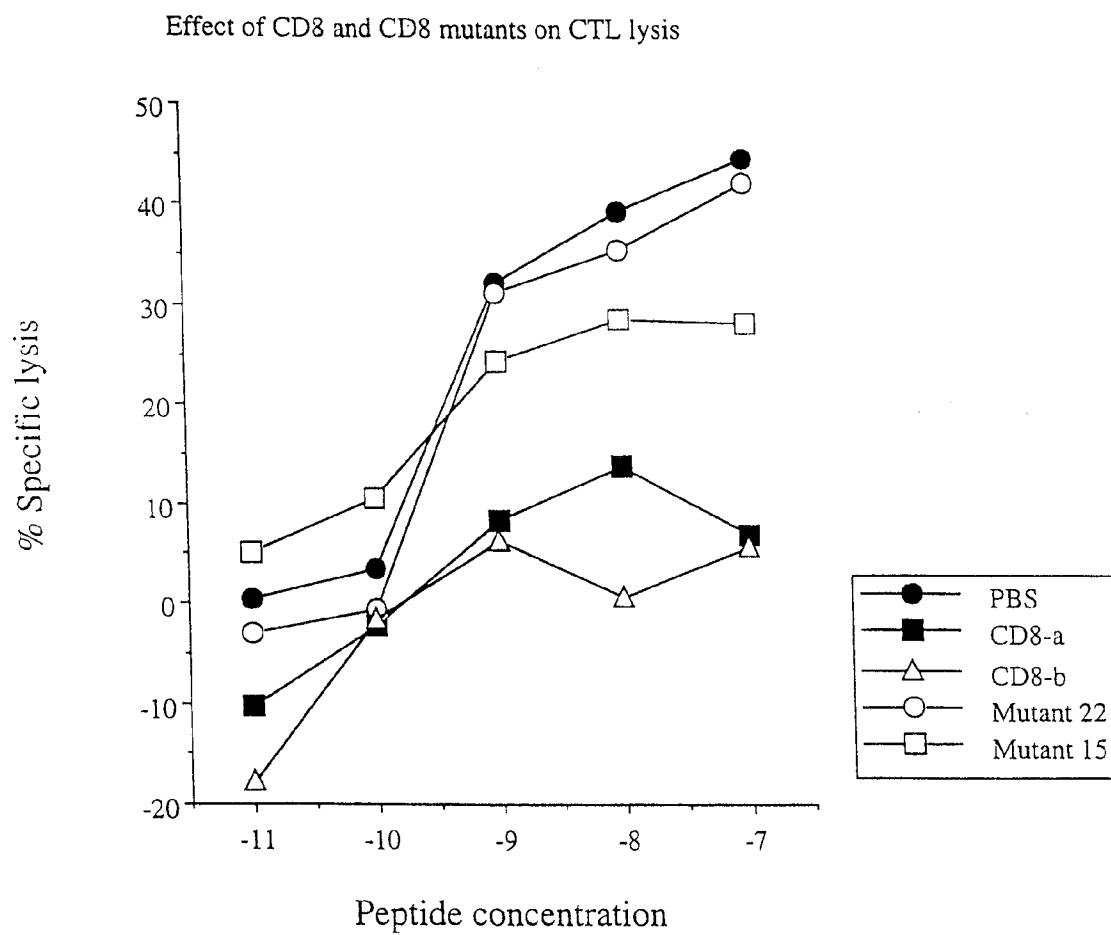
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Fig.9



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Fig.10

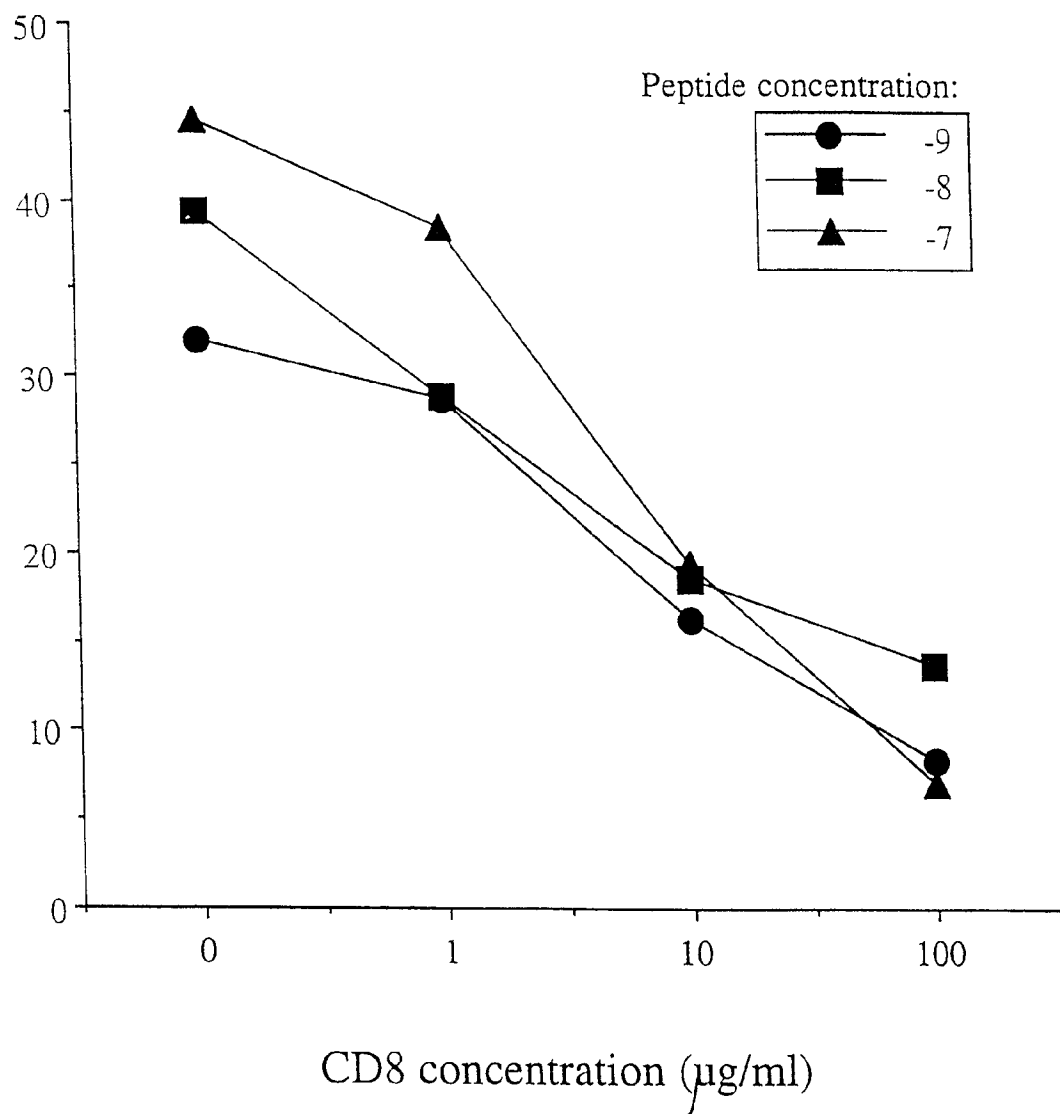




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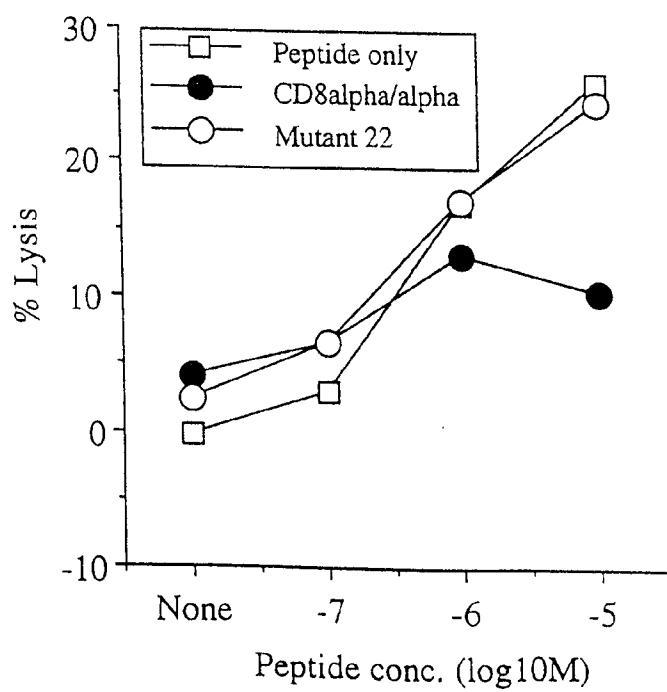
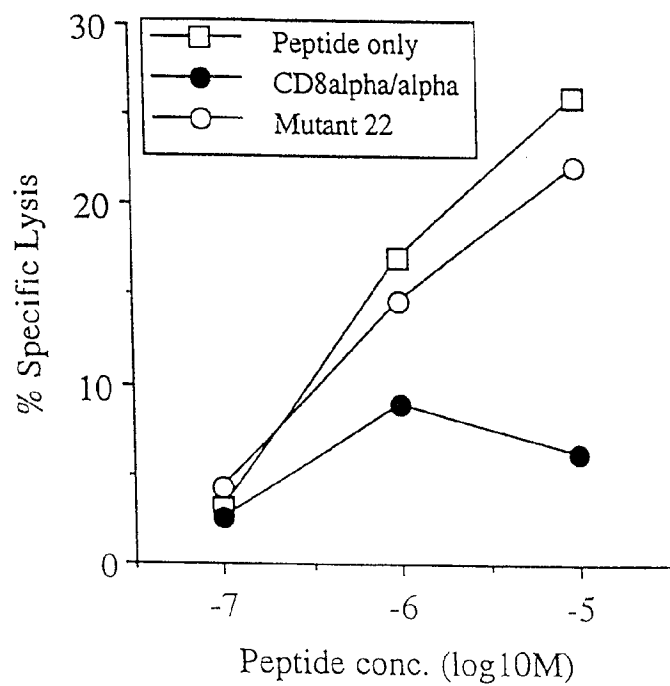
Fig.11

Effect of CD8 on CTL lysis



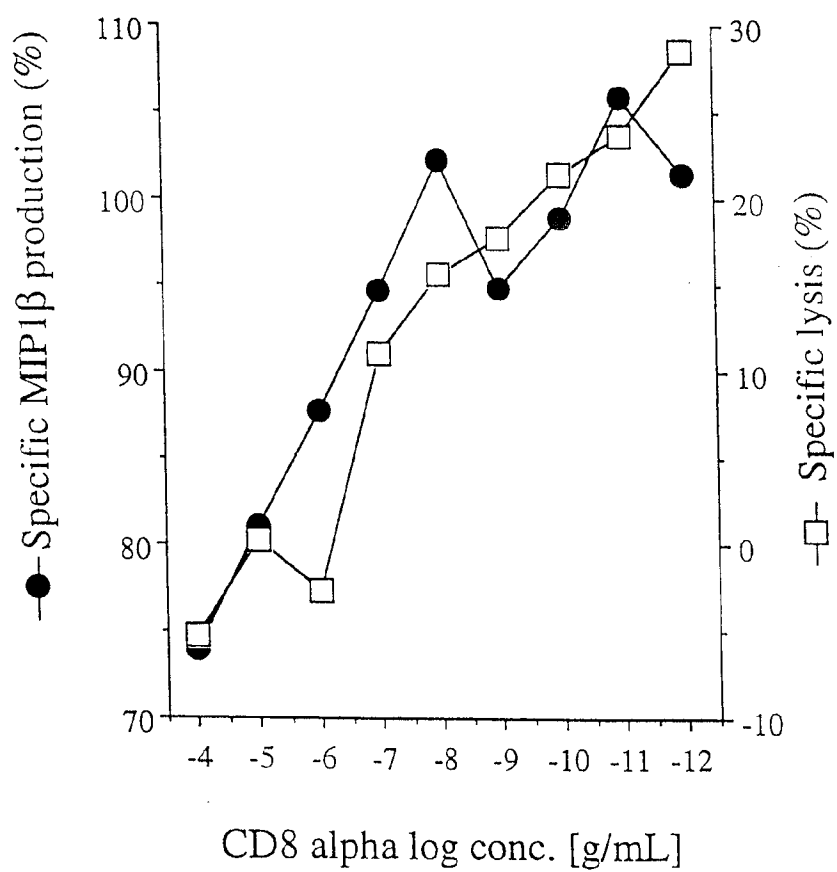
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Fig.12



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Fig.13



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Fig.14

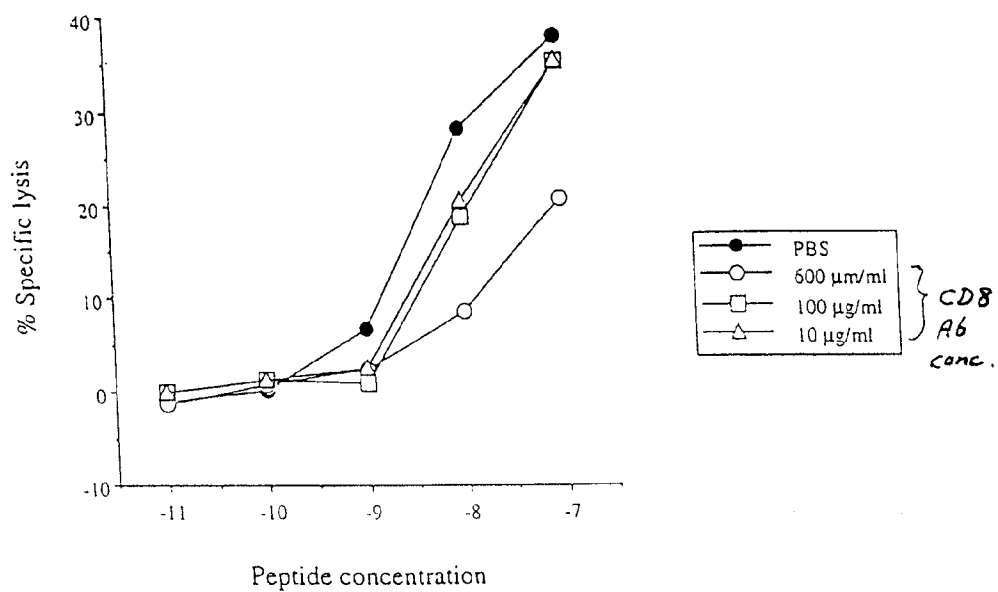
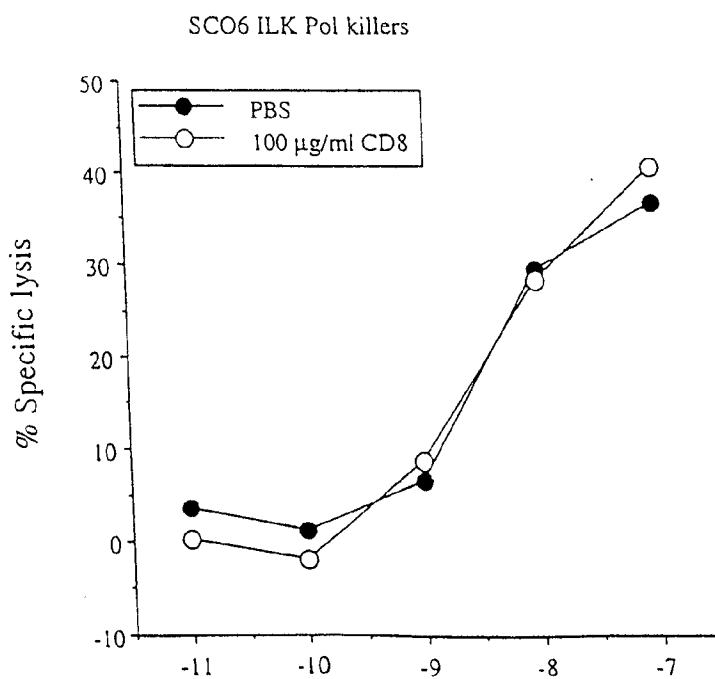


Fig.15



**Fig.16.**

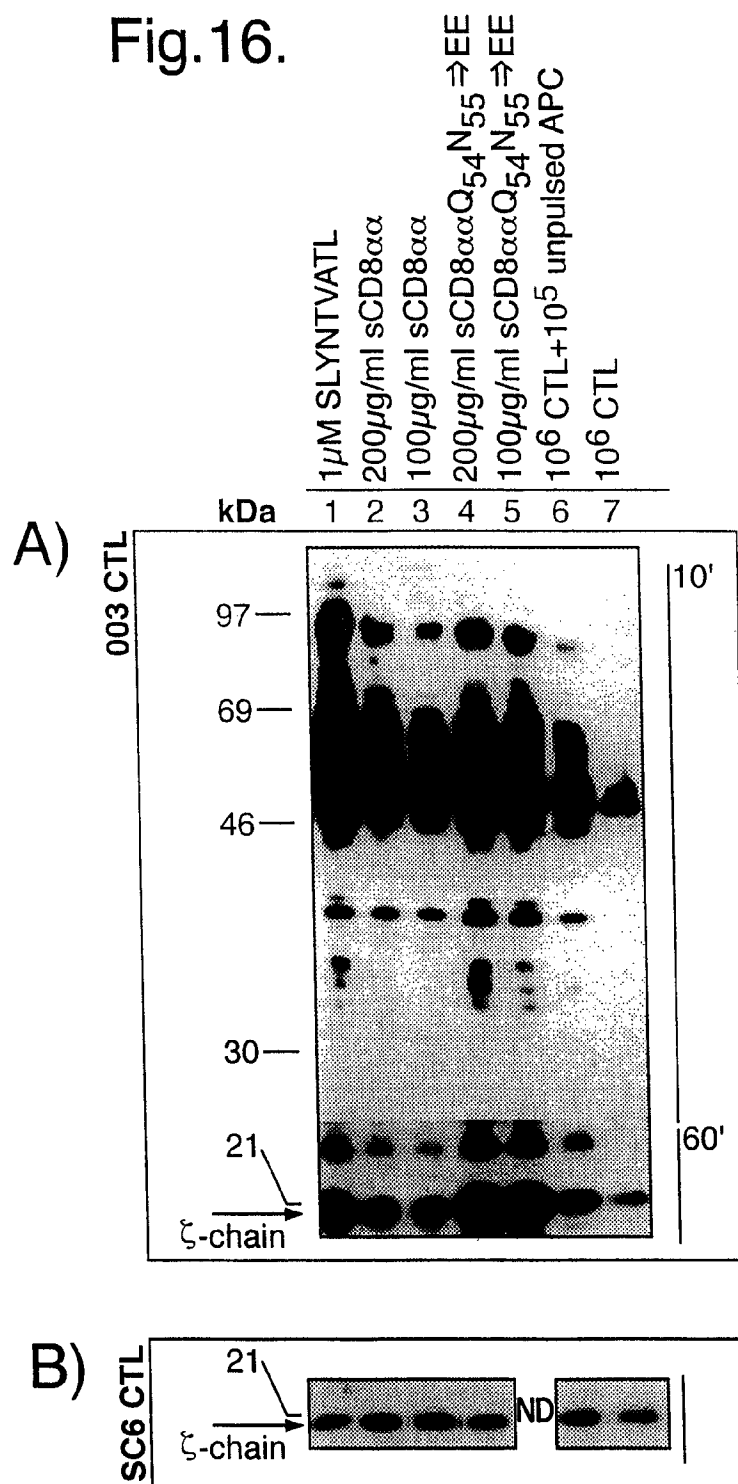
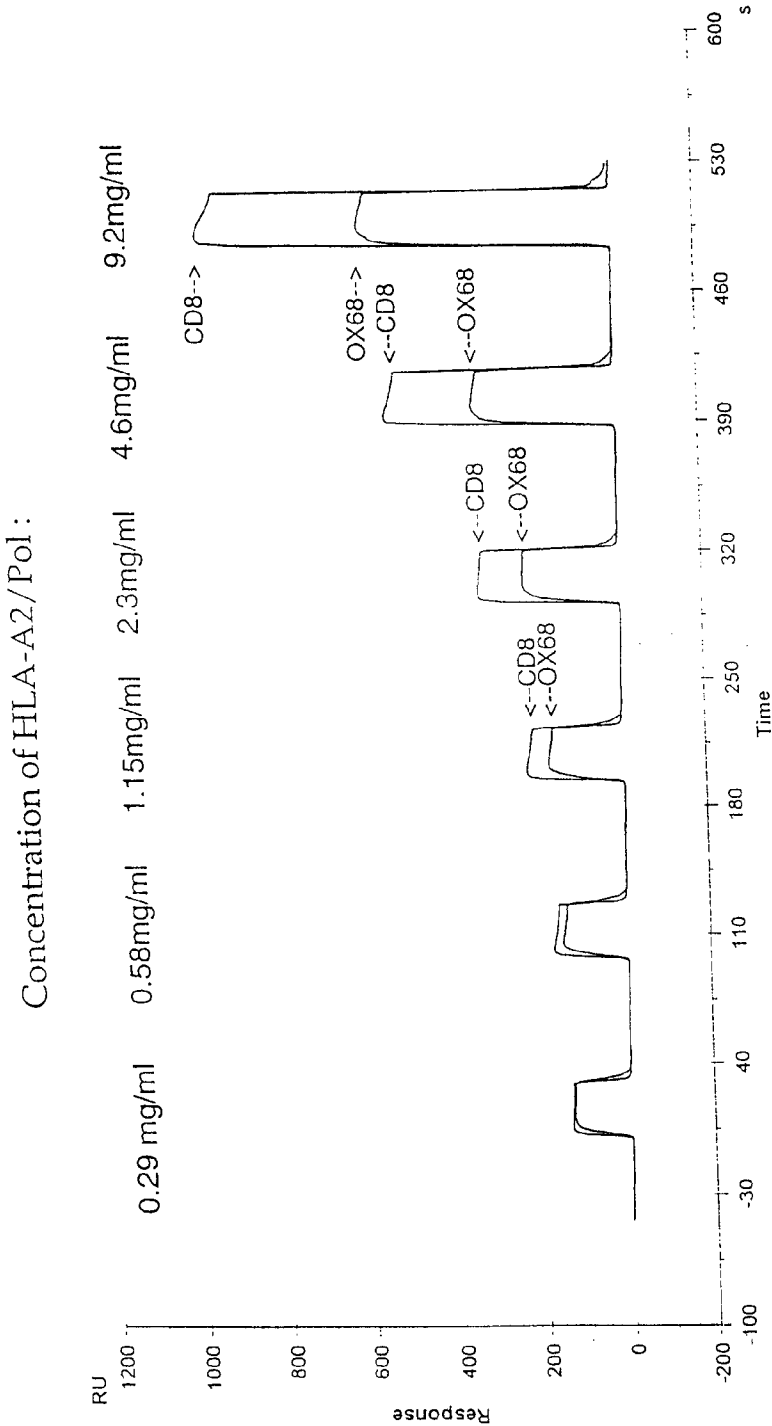


Figure 17  
Biotinylated sCD8 $\alpha$ /OX68 immobilised



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Figure 18

```
<----- signal/leader peptide-----  
M A S P L T R F L S L N L L L L G E S I  
ATGGCCTCACCGTTGACCCGCTTTCTGTGCTGAACCTGCTGCTGCTGGGTGAGTCGATT  
  
-----> <--- immunoglobulin domain -----  
I L G S G E A K P Q A P E L R I F P K K  
ATCCTGGGGAGTGGAGAAGCTAAGCCACAGGCACCCGAACCTCCGAATCTTTCCAAAGAAA  
  
----- • -----  
M D A E L G Q K V D L V C E V L G S V S  
ATGGACGCCGAACCTTGGTCAGAAGGTGGACCTGGTATGTGAAGTGTGGGGTCCGTTTCG  
  
----- # -----  
Q G C S W L F Q N S S S K L P Q P T F V  
CAAGGATGCTCTTGGCTCTTCCAGAACTCCAGCTCCAAACTCCCCAGCCCACCTTCGTT  
  
-----  
V Y M A S S H N K I T W D E K L N S S K  
GTCTATATGGCTTCATCCCACAACAAGATAACGTGGGACGAGAAGCTGAATTCGTCGAAA  
  
-----  
L F S A M R D T N N K Y V L T L N K F S  
CTGTTTTCTGCCATGAGGGACACGAATAATAAGTACGTTCTCACCTGAACAAGTTCAGC  
  
----- • -----  
K E N E G Y Y F C S V I S N S V M Y F S  
AAGGAAAACGAAGGCTACTATTTCTGCTCAGTCATCAGCAACTCGGTGATGTACTTCAGT  
  
-----> <----- stalk region -----  
S V V P V L Q K V N S T T T K P V L R T  
TCTGTGCTGCCAGTCCTTCAGAAAGTGAACCTCTACTACTACCAAGCCAGTGTGCGAACT  
  
-----  
P S P V H P T G T S Q P Q R P E D C R P  
CCCTCACCTGTGCACCCTACCGGGACATCTCAGCCCCAGAGACCAGAAGATTGTCGGCCC  
  
-----> <----- transmembrane domain -----  
R G S V K G T G L D F A C D I Y I W A P  
CGTGGCTCAGTGAAGGGGACCGGATTGGACTTCGCCTGTGATATTTACATCTGGGCACCC  
  
----->  
L A G I C V A L L L S L I I T L I C Y H  
TTGGCCGGAATCTGCGTGGCCCTTCTGCTGTCCTTGATCATCACTCTCATCTGCTACCAC
```

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<----- cytoplasmic domain ----->  
R S R K R V C K C P R P L V R Q E G K P  
AGGAGCCGAAAGCGTGTTTGCAAATGTCCCAGGCCGCTAGTCAGACAGGAAGGCAAGCCC  
----->  
R P S E K I V \*  
AGACCTTCAGAGAAAATTGTGTAA

Figure 19

Nde I

\_\_\_\_\_ M K P Q A P E L R I F P K K M D A E L  
catatgAAaCCACAaGCACcTGAACtACGAATCTTTCCAAAGAAAATGGACGCCGAACTT  
• #  
G Q K V D L V C E V L G S V S Q G C S W  
GGTCAGAAGGTGGACCTGGTATGTGAAGTGTGGGGTCCGTTTCGCAAGGATGCTCTTGG  
L F Q N S S S K L P Q P T F V V Y M A S  
CTCTTCCAGAACTCCAGCTCCAAACTCCCCAGCCCACCTTCGTTGTCTATATGGCTTCA  
S H N K I T W D E K L N S S K L F S A M  
TCCCACAACAAGATAACGTGGGACGAGAAGCTGAATTCGTCGAAACTGTTTTCTGCCATG  
R D T N N K Y V L T L N K F S K E N E G  
AGGGACACGAATAATAAGTACGTTCTCACCTGAACAAGTTCAGCAAGGAAAACGAAGGC  
•  
Y Y F C S V I S N S V M Y F S S V V P V  
TACTATTTCTGCTCAGTCATCAGCAACTCGGTGATGTACTTCAGTTCTGTCTGCCAGTC  
HindIII  
\_\_\_\_\_ L Q K V N S T T T K P \*  
CTTCAGAAAGTGAACtCTACTACTACCAAGCCAtaagctt

[SEQ ID NO: 24]



Fig.20

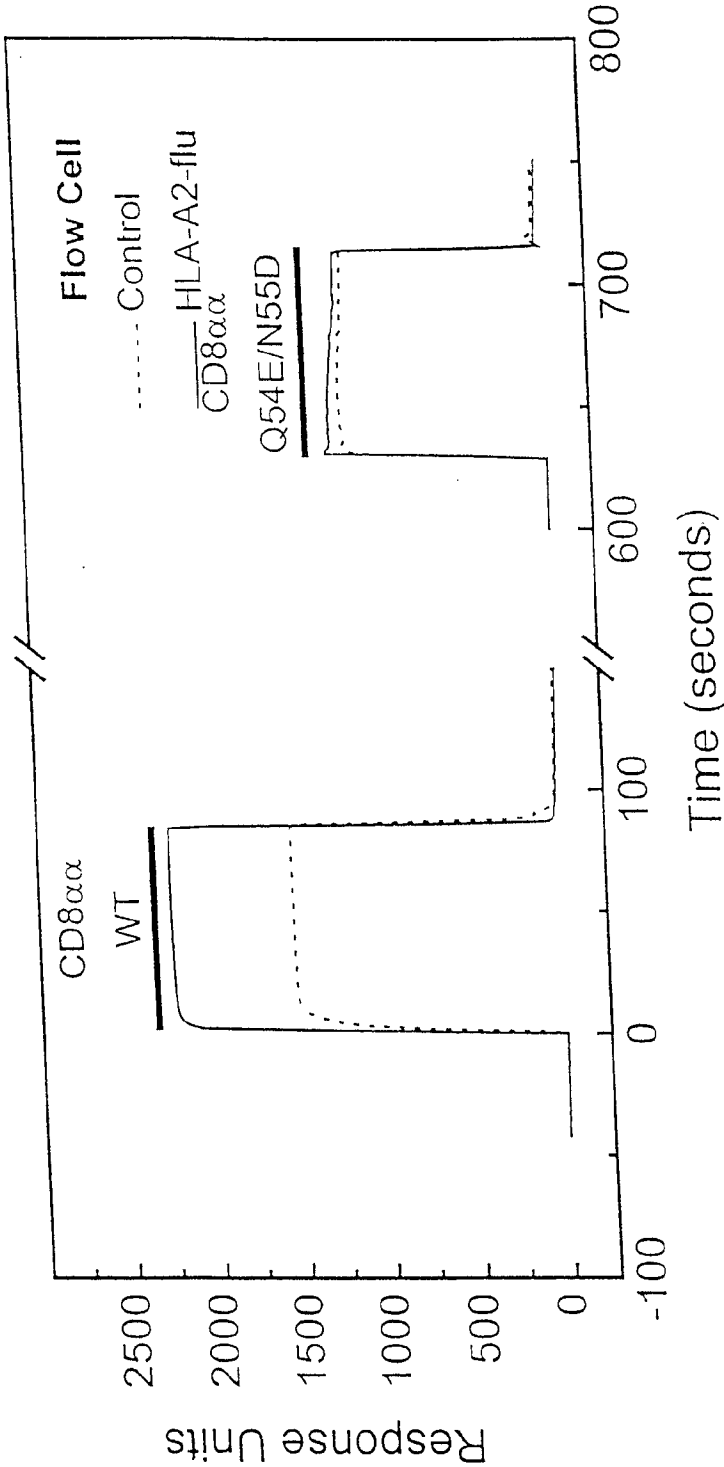
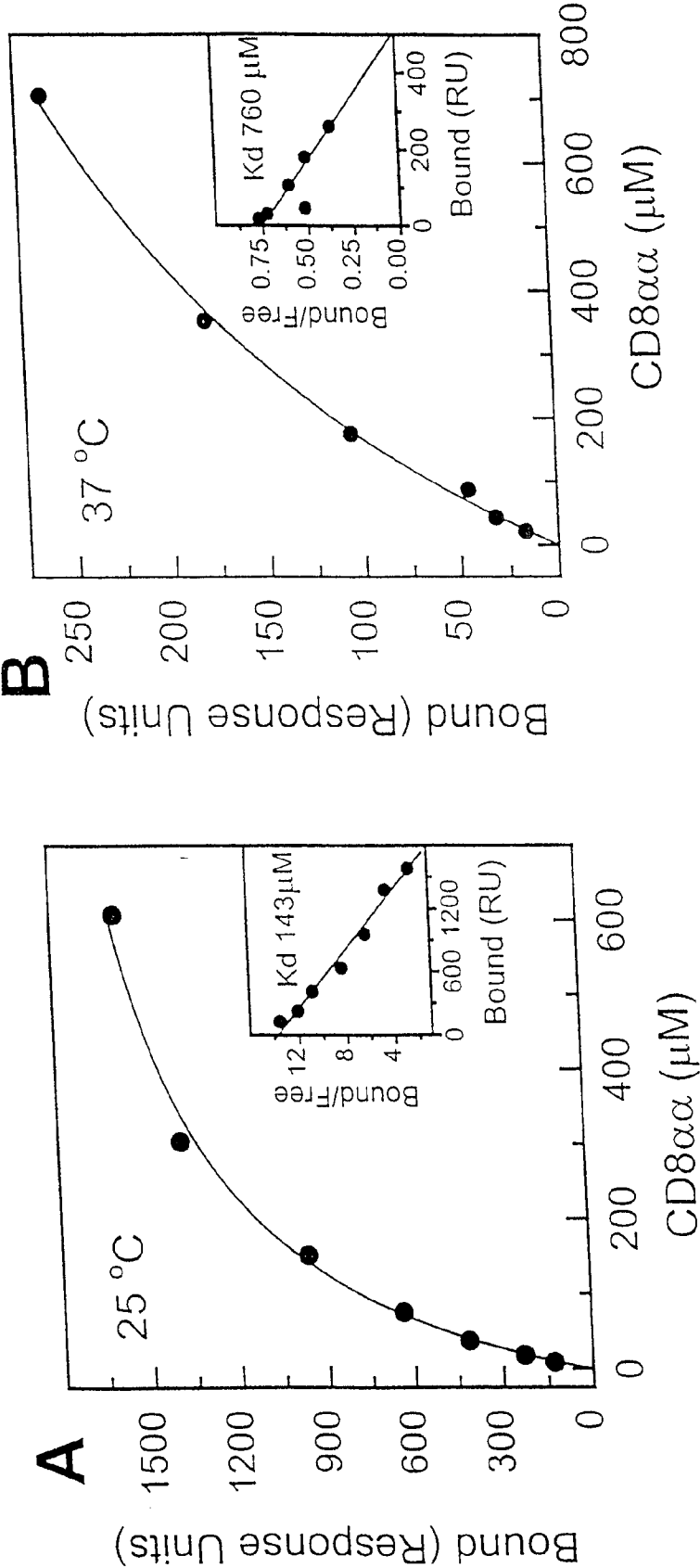
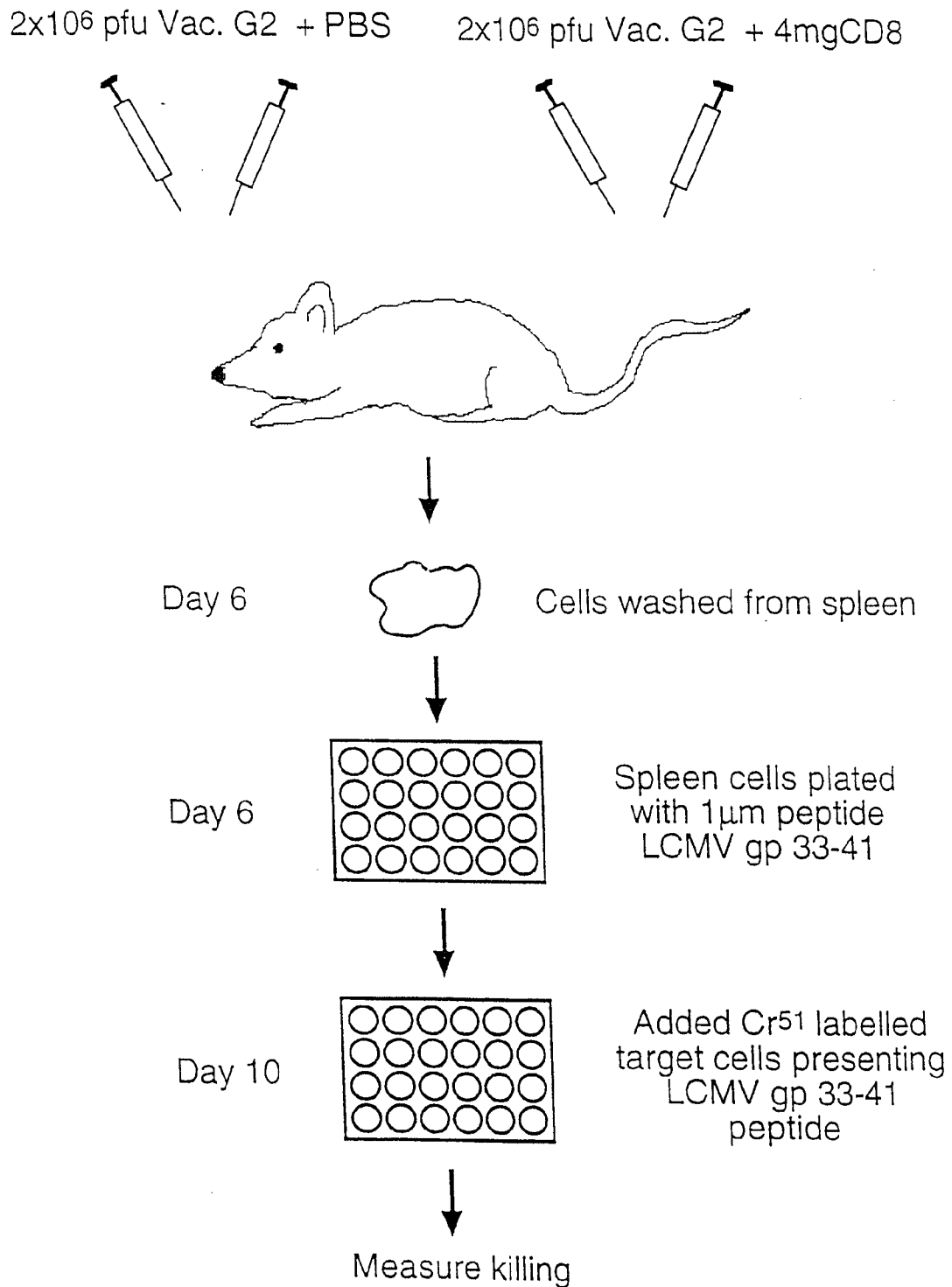


Fig.21



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Figure 22

## Human CD8 inhibition of CTL priming in vivo



# INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 98/03235

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K38/17 C07K14/705 C12N15/12

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PALIWAL V ET AL: "Recombinant soluble alphabeta T cell receptors protect T cells from immune suppression: requirement for aggregated multimeric, disulfide-linked alphabeta heterodimers." JOURNAL OF IMMUNOLOGY, (1997 AUG 15) 159 (4) 1718-27, XP002093583 see the whole document ---	1-7
A	GAO G F ET AL: "Crystal structure of the complex between human CD8alpha(alpha) and HLA-A2." NATURE, (1997 JUN 5) 387 (6633) 630-4, XP002093584 cited in the application see the whole document --- -/--	1-19



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search

16 February 1999

Date of mailing of the international search report

26/02/1999

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
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Fax: (+31-70) 340-3016

Authorized officer

Moreau, J

## INTERNATIONAL SEARCH REPORT

Intern: al Application No

PCT/GB 98/03235

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BOURSIER J P ET AL: "Evidence for an extended structure of the T-cell co-receptor CD8 alpha as deduced from the hydrodynamic properties of soluble forms of the extracellular region." JOURNAL OF BIOLOGICAL CHEMISTRY, (1993 JAN 25) 268 (3) 2013-20, XP002093585 cited in the application see the whole document ---	1-19
A	WO 96 22106 A (THOMAS JEFFERSON UNIVERSITY) 25 July 1996 see the whole document ---	1-19
P,X	GAO, GEORGE F. ET AL: "Assembly and crystallization of the complex between the human T cell coreceptor CD8alpha homodimer and HLA-A2." PROTEIN SCIENCE, (MAY, 1998) VOL. 7, NO. 5, PP. 1245-1249, XP002093586 see the whole document ---	1-7
P,X	JELONEK M T ET AL: "Direct binding of the MHC class I molecule H-2Ld to CD8: interaction with the amino terminus of a mature cell surface protein." JOURNAL OF IMMUNOLOGY, (1998 MAR 15) 160 (6) 2809-14, XP002093587 see the whole document -----	1-7

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB 98/03235

### Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.: 1-5  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 1-5  
are directed to a method of treatment of the human/animal  
body, the search has been carried out and based on the alleged  
effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such  
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all  
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment  
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report  
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is  
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

#### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 98/03235

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9622106 A	25-07-1996	US 5645837 A	08-07-1997